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Crucifer-infecting *Tobacco mosaic virus* replicase protein p122 is an RNA silencing suppressor

PhD Thesis

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Chapter I INTRODUCTION

In eukaryotes, RNA silencing serves as a sequence-specific gene inactivation system. While discovered as a side effect of transgene expression in plants and a process by which transgenic virus resistance could be obtained, it has since been implicated in natural virus resistance and important biological processes such as development, gene regulation and chromatin remodeling. Due to the biochemical dissection of components of the silencing pathways in several model organisms such as *Drosophila melanogaster, Caenorabditis elegans* or *Shizosaharomyces cerevisiae* the general understanding of how RNA silencing works has greatly increased in recent years. The revelation of a striking level of conservation of the RNA silencing pathway between most eukaryotic organisms strengthens its importance.

RNA silencing induced by double-stranded RNA molecules such as short hairpins or short interfering RNAs has developed into a standard tool in gene function studies. It is being applied in large automated genom screens, where a majority of genes of a certain organisms are knocked-down and analyzed using different assays depending on the research interests.

In plants, RNA silencing is used as a generally applicable antiviral strategy. To counteract RNA slincing-based plant defence viruses evolved silencing suppressor proteins. Characterization of these suppressor proteins not only unravel answers on pathogene-host interaction and coevolution, but gives a better insight into the mechanism of RNA silencing itself.

In this work our goal was to identify and characterize in details the silencing suppressor protein of crucifere-infecting *Tobacco mosaic virus*.

Chapter II LITERATURE REVIEW

II.1. General mechanism of RNA silencing

RNA silencing (also known as RNA interference, RNAi) is a general term for a particular collection of phenomena in which short RNA molecules trigger repression of homologous sequences. It is a highly conserved pathway, found in a large variety of eukaryotic organisms, and its main characteristic is the use of small RNA molecules of 21–25 nucleotides that confer high specificity to the target sequence. The triggers of RNA silencing are the double-stranded RNA (dsRNA) molecules, which are recognized as aberrant in the cell by RNase-like III type nucleases, the Dicers, and cleaved into small interfering RNAs (siRNA) with specific two-nucleotide 3‘ overhangs (Bernstein et al., 2001) (see Figure 1.).

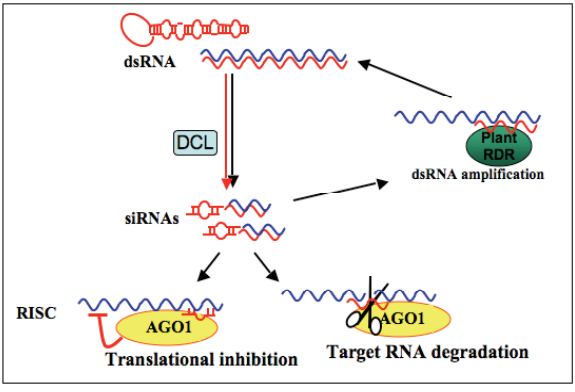

Figure 1. The RNA silencing pathway: Double-stranded RNA molecules derived from complementary transcripts or from a stem–loop structure are recognized by Dicer (in blue) and cleaved into small RNAs. The RdRP protein (in green) acts in a positive-feedback loop for the siRNA signal by producing complementary strands of the target RNA molecule, either by recognition of its ‘aberrant’ nature or by using small RNAs as primers, thus generating more homologous double-stranded RNA for Dicer processing. The central proteins of RISC complexes, the Argonautes are programmed with the small RNAs. RISC can exert silencing in a variety of forms: in all cases, the small RNA confers target specificity, whereas the protein components within the RISC complex effects cleavage of homologous RNA or recruit mediators of repression.

The siRNAs following unwinding are incorporated into multiprotein effector complexes, called RNA-induced silencing complexes (RISC) (Pham et al., 2004). RISCs target homologous

RNAs and exert silencing on post-transcriptional level, either by inducing cleavage ('slicing') or by blocking translation.

RNA-dependent RNA polymerase (RdRP) also plays a role in nematodes, plants and fungi but is apparently not required or detectable in the genomes of flies and vertebrates (Vazquez, 2006). RdRP amplifies the RNAi response by generating more double-stranded RNA from single-stranded targets that can then enter and continue to stimulate the RNA silencing pathway. This positive-feedback system is crucial to amplify the siRNA signal transmitted from cell to cell and to mount a systemic form of silencing.

II.2. Brief history of RNA silencing

The first observation of RNA silencing was reported be van der Krol, Napoli and their coworkers when they was unable to over express chalcone synthase (CHS) in transgenic petunia plants. In order to get an increase in flower pigmentation, petunia flowers were transformed with the CHS gene using different constructs that should have led to over expression. Instead the opposite effect was observed: some of the plants showed patchy or reduced pigmentation and others were completely white. It was shown that when extra copy or copies of the transgene was present the CHS mRNA level was reduced in the white sectors. Since the transgene suppressed both its own and also the endogenous gene expression the phenomena was called co-suppression. Not much later, another encounter with RNA silencing was made in the field of virus resistance by several groups. In different viral systems was demonstrated that in contrast to the original model, the expression of viral proteins was not required for viral resistance, but untranslatable viral RNA was sufficient for establishing pathogen-derived resistance (PDR) (Lindbo et al., 1992; van der Vlugt et al., 1992). The observation that a silenced GUS transgene could prevent virus accumulation of *Potato virus X* (PVX) carrying GUS sequences pointed toward a role in a sequence specific antiviral defense mechanism, what was than called post-transcriptional gene silencing (PTGS) (English et al., 1996). Supporting evidence of the more general nature of this plant response to viral infection was provided by the finding that the plant would not only be resistant against the initially inoculated virus but would be cross-protected against other viruses carrying homologous sequences (Ratcliff et al., 1999). These phenomena are now generally known as virus-induced gene silencing (VIGS). To explain the extreme sequence specificity of the RNA silencing process, small RNA molecules had

been envisaged in models after the report of Hamilton and Baulcombe (Hamilton & Baulcombe, 1999) who unequivocally proved that plants containing a silenced transgene indeed accumulated small double-stranded RNA molecules approximately 25 bp length, whose sequence was identical to the transgene. They also observed the same sequence-specific small RNA pool in PVX infected plants, suggesting a more general role of these molecules. A further breakthrough pointing to the involvement of RNA silencing in antiviral defense was the discovery of virus specific RNA silencing suppressors (discussed later in more details). To increase the generality of RNA silencing the next step was achieved in animal research by Fire and coworkers (Fire et al., 1998). At that time sense and anti-sense transcripts were already being used to knock-down gene expression in *C. elegans*. The real breakthrough came when they injected very low amounts of double-stranded RNA (dsRNA) (used as control experiment) and this induces more efficiently RNA silencing than just using single-stranded sense or anti-sense RNAs. Later became clear that the building units of the gene silencing are very similar in the different organisms and therefore suggest an ancient role.

II.3. The biochemistry of RNA silencing

RNA silencing of endogenous genes, viruses, and selfish genomic elements is a regulatory process that relies on small RNA molecules, approximately 21-25 nucleotides long (Hamilton & Baulcombe, 1999, Kim, 2005). The trigger of RNA silencing is an RNA molecule harboring a duplex region (see Figure 1.). Such a molecule is processed with the following steps: (i) small RNA production: a precursor RNA is cleaved to produce small dsRNAs, where the precursor can be a hairpin-structured RNA in the case of microRNA (miRNA) or a long dsRNA for different types of small interfering RNA (siRNA); (ii) ribonucleoprotein (RNP) complex assembly: after unwinding one strand of small RNAs is loaded into an RNP (mature strand), the other strand rapidly degraded (star strand) (Schwarz et al., 2003, Khvorov et al., 2003), and (iii) gene silencing: the RNP suppresses its target gene, where the target recognition is guided by the loaded small RNAs, and the silencing activity is mediated by the proteins composing the RNP at the post-transcriptional or transcriptional level (Bartel, 2004, Almeida et al., 2005, Brodensen et al., 2008, Eamens, A., et al., 2008). This machinery is adopted in a wide range of organisms. Although the overall pathways resemble each other, there are substantial differences between organisms.

Dicer and Drosha proteins: In metazoans, two RNase III endonucleases, Drosha and Dicer, contribute to a process of small RNA production. Drosha cleaves a long primary transcript including a stem-loop (termed primary miRNA, or pri-miRNA) near the base of the stem to release a hairpin structure, termed precursor miRNA (pre-miRNA). Dicer cleaves pre-miRNA or a long dsRNA (precursor of siRNA) to produce a small dsRNA, only one strand of which is loaded into the RNP (Bartel, 2004; Hutvagner et al., 2002). *Homo sapiens, Mus musculus, and Caenorhabditis elegans* have only one Dicer gene, which contributes to both miRNA and siRNA production, however in several organisms these two roles are encoded by distinct genes. *Drosophila melanogaster* has two proteins of Dicer, DCR-1, and DCR-2, which are used for miRNA and siRNA, respectively (Kavi et al., 2005). *Arabidopsis thaliana* has four Dicer orthologues, DCL1 to DCL4, but no Drosha. DCL1 contributes to miRNA production, DCL2 to 22nt siRNA from invading viruses, DCL3 to 24nt siRNA from endogenous genes (Bonnet et al., 2006; Herr, 2005) and DCL4 to 21nt trans-acting RNAs (tasiRNA) (Gasciolli et al., 2005).

The double-stranded RNA binding proteins (DRB): Dicers are associated with double-stranded RNA-binding proteins (DRBs). In plants there are several potential DRBs. DCLs act redundantly and hierarchically, but there is little or no redundancy or hierarchy amongst the DRBs in their DCL interactions. HYPONASTIC LEAVES1 (HYL1) is a DRB protein which cooperates with DCL1 and required in processing of microRNA (miRNA) precursors in the plant cell nucleus. DCL4 operates exclusively with DRB4 to produce trans-acting (ta) siRNAs and 21nt siRNAs from viral RNA. DCL2 and DCL3 produce viral siRNAs without requiring assistance from any dsRBP. DRB2, DRB3 and DRB5 appear unnecessary for mi-, tasi-, viral si-, or heterochromatinising siRNA production.

The Argonautes: There are also differences in the effector complexes participating in RNA silencing. Their main components are the Argonaute proteins, which have two principal domains: an RNA-binding PAZ domain at the N-terminus and RNase-like Piwi domain at the C-terminus. The Argonaute protein family consists of the Ago subfamily, the Piwi subfamily, and the *C. elegans* specific subfamily (Parker et al., 2006). Mammalian AGO subfamily members contribute to both the siRNA and miRNA pathways. In contrast, AGO1 contributes only to the miRNA pathway, and AGO2 only to the siRNA pathway in *D. melanogaster* (Kavi et al., 2005). The many members in *C. elegans* have also been suggested to have a distinction in their roles (Yigit et al., 2006). Intriguingly, the Piwi subfamily is not found in *A. thaliana*. This organism has only AGO subfamily

members, where AGO1 is involved in miRNA and endogenous siRNA, and AGO4 in DNA methylation through endogenous siRNA (Herr, 2005).

The RdRP proteins: The amplification of small RNAs relying upon RdRP proteins. The polymerase synthesizes dsRNAs from RNAs cleaved by siRNA or miRNA programmed RISC (siRISC or miRISC respectively), and the synthesized dsRNAs are used as siRNA precursors (Nishikura, 2001) (see Figure 1.). This process contributes to the amplification of siRNA and the subsequent silencing effect. In plants, two cleavage events often trigger siRNA biogenesis by this amplification pathway (Axtell et al., 2006, Lipardi et al., 2001).

The combination of various members of the DCL, RDB, AGO and RdRP gene families contribute in the parallel silencing pathways, including the microRNA (miRNA), trans-acting siRNA (tasiRNA), natural-antisense siRNA (natsiRNA), and repeat-associated siRNA (rasiRNA)/RNA-directed DNA methylation (RdDM) pathways. Figure 2 gives a schematic representation of the sequential steps involved in the parallel pathways of plant gene silencing.

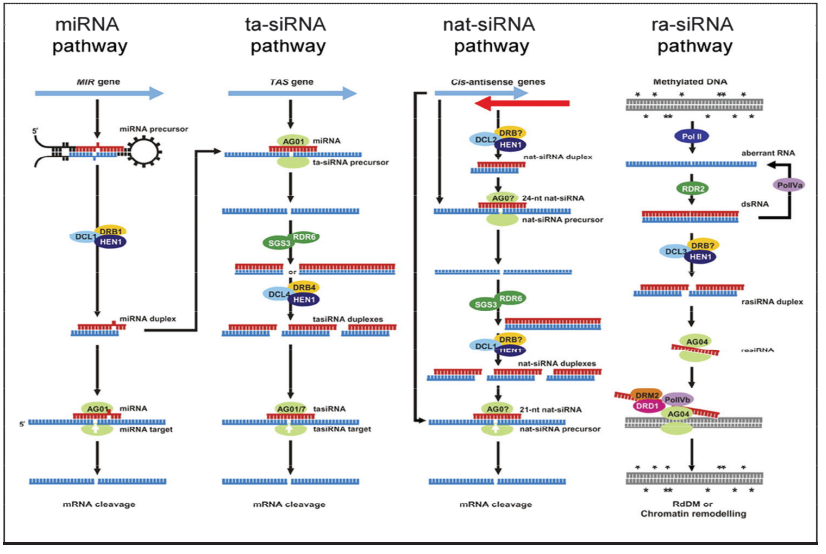

Figure 2: The parallel silencing pathways of *A. thaliana.* Eamens, A., et al. Plant Physiology. 2008

The miRNA pathway in plants: miRNAs are processed from single-stranded RNA transcripts (transcribed from MIR genes), the primary-miRNA transcript is cleaved to produce the shorter precursor-miRNA (pre-miRNA) and in a second step the miRNA duplex (miRNA/miRNA*), by the combined action of DCL1 and HYL1 (Vazquez et al., 2004). The two-nucleotide 3' overhangs of the liberated miRNA duplex are methylated by the sRNA-specific methyltransferase HUA ENHANCER1 (HEN1) (Li et al., 2005). The methylation was proposed to stabilize siRNAs and miRNAs against uridinylation and subsequent degradation. The pre-miRNAs or miRNAs are transported into the cytoplasm mainly by an active transport mechanism involving HASTY (HST) (Park et al., 2005), the Arabidopsis orthologues of Exp5. In cytoplasm the miRNA duplex is unwinded: based on thermodynamic properties (Schwarz et al., 2003, Khvorova et al., 2003) is chosen the mature single-stranded miRNA and loaded onto AGO1, the catalytic center of plant RISC, the other strand, the miRNA star (miRNA*) is quickly degraded. The mature miRNA guide the slicer activity of AGO1 to repress the expression of complementary mRNAs, and in plants, miRNA-directed repression of gene expression is predominantly mediated by transcript cleavage (Roades et al., 2002, Baumberger et al., 2005, Brodensen et al., 2008).

The ta-si pathway: Two miRNAs, miR173 and miR390, have been shown to induce an additional level of complexity to the control of gene expression for normal development in plants (Axtell et al., 2007). These miRNAs bind to their target tasiRNA transcripts (TAS), directing cleavage of the TAS transcript in a miRNA/DCL1/HYL1-mediated manner. However, instead of becoming silenced, these cleaved noncoding RNA transcripts are used as templates for dsRNA synthesis by the RDRP, RDRP6, with the help of the coiled-coil protein, SUPPRESSOR OF GENE SILENCING3 (SGS3). The dsRNA is then processed into phased 21-nucleotide tasiRNAs by DCL4 in a sequential process initiated at the miRNA cleavage site. Similar to DCL1/HYL1 processing of MIR-derived hairpin RNAs (hpRNA), DCL4 functions in tandem with the dsRBP, DRB4, to generate the phased tasiRNA from RDRP6/SGS3-generated TAS dsRNA. The tasiRNAs then target their own specific cognate mRNAs for degradation (Howell et al., 2007).

The nat-siRNA pathway: The *A. thaliana* genome encodes more than 2,000 natural-antisense gene pairs, and these endogenous cis-antisense genes are transcribed from different DNA strands to produce dsRNA transcripts that harbor regions of complementarities at their 3' ends (Borsani et al., 2005). The dsRNA molecule formed by these complementary end sequences provides a substrate

for DCL2 cleavage and the generation of a single 24-nucleotide natsiRNA. This single 24-nucleotide natsiRNA subsequently targets one of the cis-antisense gene pair transcripts for cleavage, and the cleaved RNA molecule is converted to dsRNA by RDRP6 and SGS3. The RDRP6/SGS3-synthesized dsRNA molecule is then processed into phased 21-nucleotide natsiRNAs by the action of DCL1. The phased 21-nucleotide natsiRNAs, like the tasiRNA class of endogenous small RNAs, are in turn used as guides to direct sequence-specific silencing of homologous mRNAs.

The ra-siRNA pathway Another RNA silencing-related pathway in *A. thaliana* that is regulated at the sRNA level is transcriptional gene silencing (TGS), which is an epigenetic mechanism resulting in the silencing of a transgene or an endogenous gene through the inactivation of their promoter sequences. DNA methylation is essential for normal plant and animal development and is also a hallmark of TGS (Mette et al., 2000). In fact, the majority of methylation in plants is associated with repeat sequences, such as transposons, and methylation of these sequences is thought to occur as a natural suppressor to control their expression (Wassenegger, 2005). In *A. thaliana*, repeat sequences have been shown to be the source of a unique class of siRNAs, termed rasiRNAs, which are of the 24-nucleotide size class, and rasiRNAs have been suggested to direct DNA methylation and hence to transcriptionally silence repetitive DNA sequences in the plant genome (Chan et al., 2005). Methylated DNA is thought to act as a template for the transcription of aberrant RNA. The transcription is done by either RNA polymerase II (PolII), or the plant-specific Polymerase IVa enzyme (PolIVa). This aberrant RNA is then converted to dsRNA by RDRP2 or PolIVa and processed by DCL3 into 24-nucleotide siRNAs that are methylated by HEN1 and used by AGO4 to direct the actual sequence-specific DNA methylation step by RNA dependent DNA-methyltransferase (RdDM) and subsequent heterochromatinization of DNA (Kanno et al., 2005).

II.4. Functions of RNA silencing

The biochemical machinery of RNA silencing support several processes. There are two forms of RNA silencing: transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS). These two forms support different functions. Among these processes are transposon silencing due to sequence specific DNA methylation and chromatin condensation (TGS), developmental gene regulation, stress responses and antiviral defense (PTGS).

Transposon silencing

One of the first indications that RNA is involved in transcriptional gene silencing (TGS) in the nucleus was done by Wassenegger et al. (1994). Upon viroid infection of plants transformed with T-DNAs containing viroid cDNA sequences, the latter became methylated while the other part of the T-DNA were unaffected. They conclude that the replicating viroid RNA leads to specific methylation of homologous sequences in plant genom. The phenomenon was named RNA-dependent DNA methylation (RdDM). Expression of dsRNA of promoter sequences was shown to be a trigger for sequence-specific RdDM of these promoters and subsequently induced transcriptional gene silencing (TGS) (Mette et al., 2000). The fact that promoter-derived dsRNA was processed into siRNAs suggested a role for this molecules in the TGS in the nucleus. Endogenous repeat-associated small RNAs trigger de novo methylation of homologous DNA and by this contribute to heterochromatin formation (Xie et al. 2004). Lately several components of the RdDM pathway were identified. The other role of methylation beside TGS is the chromatin remodeling discovered in *Schizosaccharomyces pombe* (Volpe et al., 2003). Three genes that encode key enzymes of the RNA silencing machinery, Argonaute, Dicer and an RDRP were shown to be essential for this process: the RDRP produce dsRNA from transcripts originating from pericentromeric heterochromatin composed of repeat sequences, these duplexes are than rapidly processed by Dicer into siRNAs which are incorporated into RNA-induced transcriptional gene silencing (RITS) complex. This complex is highly similar to RISC, having the same core component, one of the Argonaute family proteins, in *S. pombe* Ago1 (Verdel et al., 2004). RITS activity leads to methylation of the heterochromatic regions and ultimately to chromosome condensation (Noma et al., 2004). These processes are conserved among eukaryotes.

Developmental gene regulation

One of the recent major discoveries in developmental biology was the finding that the eukaryotic organisms produce endogenous small RNAs, which turned out to be of huge impact as they influences gene expression in an unforeseen way and scale. The most important group of this endogenous small RNAs are the micro-RNAs (miRNAs). They are phylogenetically conserved across species. Typically they are encoded in the genom as more or less imperfect hairpins as part of larger processed transcripts. These are incorporated into RISC complexes and guide it for the cleavage or inhibition of translation of homologous mRNAs. Most plant miRNAs studied so far have a near perfect complementarities to their target in the open reading frame (ORF) leading to

mRNA cleavage (Rhoades et al., 2002), however a recent paper provides evidence that plant miRNA–guided silencing has a widespread translational inhibitory component that is genetically separable from endonucleolytic cleavage activity even if the complementarity between the miRNA and mRNA is near perfect (Brodensen et al., 2008). Translational inhibition by miRNAs but not cleavage was described in the case of APETALA2 transcript (Aukerman et al., 2003), while this is the main mode of action in animals (Ambros, 2004).

A great number of genes have been identified as being regulated by the miRNAs. It was found that many predicted targets are transcriptional factors involved in development (Roades et al., 2002, Kidner et al., 2004). miRNAs also targets genes involved in abiotic stress (Sunkar et al., 2004) and a variety of biological process including cell proliferation, differentiation, apoptosis immune responses and stem cell functions (Lindsay, 2008).

Antiviral function of RNA silencing gene

Early studies in plants pointed to a role for silencing pathway as a defense against viruses. The first indications that RNA-mediated responses play an important antiviral role came from observations that transgenic expression of viral sequences protected plants from homologous viruses by conferring sequence-specific degradation of viral RNAs (Linbdo et al., 1992). Later it was shown that sequence-specific RNA degradation was a natural plant antiviral response (Ratcliff et al., 1997). Most known plant viruses have RNA genomes and replicate via dsRNA intermediates, thereby serving as potent inducers of RNA silencing early in replication and as silencing targets later in infection. Moreover, viral proteins were identified that suppress RNA-mediated defense (Kasschau & Carrington 1998), indicating that pathogens have evolved efficient counter defensive strategies. These viral suppressors are powerful tools to help unravel the mechanism of RNA silencing in plants.

II.5. Mounting the plant antiviral defense

As most plant viruses are RNA viruses that replicate via double-stranded replication intermediates, it is tempting to think that these molecules are the trigger for RNA silencing. However the situation is more complex: the chance that these RNAs are present in a naked form is very small, since replication complexes are protected by viral replication complexes and capsid proteins. Viral replication takes place inside specialized replication structures and the viral dsRNA

can immediately be unwound by viral or host RNA helicases (Ahlquist, 2002). Nevertheless it is possible, that these structures are detected by the silencing machinery. Our group showed evidences that more likely the virus RNA could be recognized by the plant in single-stranded form as being aberrant and directly chopped by Dicer into siRNAs. These molecules are imperfect duplexes having a non-random distribution along the viral genom, and they map asymmetrically mostly to the positive strand of the virus (Molnár et al., 2005).

Upon virus infection the primary viral siRNAs originating directly from the virus can subsequently be used in two ways: either after unwinding one strand is incorporated into the RISC to target and degrade RNAs homologous to the siRNAs (Pantaleo et al., 2007), or plant RDRP uses the siRNAs as primers on homologous mRNAs and synthesizes dsRNA that is then processed by DCLs into secondary siRNAs in a phenomenon called transitivity (Vastij et al., 2002). This latter step leads to the amplification of the silencing signal (Figure 3). It cannot be exluded that viral-siRNA-loaded RISC complexes can inhibit translation of viral RNAs.

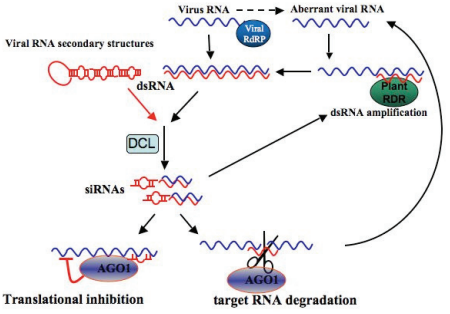

Figure 3. Antiviral silencing in plants: viral ssRNA with strong secondary structures or replicative intermediate (dsRNA) is recognized by DCL and chopped into siRNAs, which can take two ways. They can enter into amplification processes mediated by RDRP complexes, and hence produce more siRNA or can be incorporated into RISC complexes (the central protein of RISC is Argonaute (AGO)) and induce the effector step either degrading (Pantaleo et al., 2007) or translationally inhibiting (not proved yet) homologous RNA molecules.

Next to the predominant 21 nt species of siRNAs observed in all eukaryotes, the plant silencing machinery has the unique ability to produce a second size class of siRNAs of about 24 nt

(Hamilton et al., 2002). The shorter class are though to act in cell-autonomous silencing and to move from cell to cell spreading no further than up to 15 cells (Himber et al., 2003), the longer class has been proposed to correlate with the long-distance spread of RNA silencing (Hamilton et al., 2002, Dunoyer et al., 2007).

II.6. Viral countermeasures against RNA silencing: suppressor proteins

Now is commonly accepted the interpretation of the major role of RNA silencing: to provide antiviral defense. As a defensive countermeasure, viruses have evolved the capacity to encode suppressors of RNA silencing. This was first shown to be the case for plant viruses, latter animal-infecting viruses were also found to encode suppressors of RNAi (Silhavy & Burgyán, 2004; Baulcombe et al., 2004; Li & Ding, 2005). Many of the suppressors identified to date can be considered multifunctional because they also perform additional roles during the virus life cycle, for example, by functioning as a component of the replicase complex, as coat protein (CP), as movement protein (MP), or as an insect transmission factor (Scholthof, 2005).

Experiments to determine the biochemical mode of action of virus-encoded suppressors are currently a highly active area of research. Thus far it is known that suppressors can target distinct processes in the silencing pathway. Some suppressors seem to strictly and highly effectively target a specific step, like the sequestration of 21-nt siRNAs by P19 (Vargason et al.,2003; Lakatos et al., 2006) or degradation of Argonaute protein by P0 (Baumberger et al., 2007, Bortolamiol et al., 2007). Others may target more than one event; for instance, 2b interacts with dsRNA (Goto et al., 2007) and Ago1 (Zhang et al., 2006) or TCV CP and P14 bind both to siRNA and to long dsRNA (Merai et al., 2006). Recently it was also observed that suppressors may prevent key siRNA methylation steps (Vogler et al., 2007, Lózsa et al., 2008). Irrespective of the precise mechanism, all virus-encoded suppressors appear to share the overall ability to compromise RNAi-mediated degradation of viral RNA genomes or transcripts.

II.7. Crucifer-infecting *Tobacco mosaic virus* (cr-TMV): the model system

As an experimental system *Tobacco mosaic virus* (TMV) (Tobamovirus genus, discussed later) has earned his reputation as a workhorse for many areas of biology, including plant pathology being used to study and understand the general mechanisms of host-pathogen interactions (Scholthof, 2004). From a practical viewpoint the properties of TMV make it a good model system: it rapidly accumulates to high titers in infected plants, it is not transmitted by insects, fungi or nematodes, but is easily transmitted by rub-inoculation, and TMV symptoms are easy to identify on infected plants. The virus also is stable for years or even decades under ideal conditions. Another important consideration is the host range: TMV readily infects tobacco and other solanaceous plants and upwards of 200 other species.

Tobamoviruses constitute a group of plant viruses having single positive-sense RNA molecules as genomes and forming rod-shaped virions consisting of RNA and coat protein (CP) molecules. The genome is capped at the 5' end and has a tRNA-like structure at the 3' end. Characterized tobamoviral genomes encode four polypeptides. Two polypeptides are translated beginning at the same 5 '-proximal initiation codon and function in viral RNA replication. The longer is formed by suppression of termination at a single UAG codon near the middle of the genome. The shorter polypeptide contains motifs identifying it as a methyltransferase (MT) active in RNA capping and an RNA helicase (Hel). The longer polypeptide contains, in addition, motifs identifying the C-terminal domain as an RNA-dependent RNA polymerase (RDRP). A polypeptide required for intercellular movement of the virus (movement protein, MP) and the CP polypeptide are translated from subgenomic mRNAs derived from the 3' part of the tobamoviral genomes.

Tobamoviruses have been classified into three subgroups (Lartey et al., 1996). For subgroup 1 tobamoviruses (most of which infect solanaceous plants: *Tobacco mosaic virus*, strains vulgare (TMV-U1) and Ob (TMV-Ob), *Tomato mosaic virus* (ToMV), *Pipper mild mottle virus* (PMMV), *Odontoglossum ringspot virus* (ORSV)), the origin of assembly is located within MP ORF, whereas for subgroup 2 tobamoviruses (isolated from cucurbits and legumes: sunn-hemp mosaic virus(SHMV), cucumber green mottle mosaic virus (CGMMV)) the site is within CP ORF. The subdivision of this two class is supported by phylogenetic analysis of gene sequence, peptide fragmentation pattern, aminoacid composition and gene organization. A third subclass of tobamoviruses are those isolated from crucifers (*Ribgrass mosaic virus* (RMV), *Turnip vein-*

clearing virus (TVCV), *Crucifer-infecting tobamovirus* (cr-TMV), *Oilseed rape mosaic virus* (ORMV)). These are distinct in amino acid sequence and genom organization. The MP and CP ORFs overlap by 77 nucleotides (see Figure 4A).

For our study we have chosen the crucifer infecting strain of tobamoviruses, the cr-TMV, since it is able to infect *A. thaliana* the model plant of biology. The genomic RNA of cr-TMV directs the translation of a 122kDa protein and its read-through product, a 178kDa protein, which are involved in the replication of the viral RNA. The other two proteins, the 29kDa movement protein and the 18kDa coat protein are translated from individual 3'coterminal subgenomic RNAs (Dorokov et al., 1994) (Figure 4B). Several lines of evidence support the involvement of 122kDa and 178kDa in the replication of viral RNA. Both proteins have methyltransferase and helicase activity, the p122 protein is composed of three domains: a methyltransferase 1 domain (MT) 42-466 aa, an intervening region (IR) and a helicase domain (HEL) 823-1076 aa, whereas the 178K protein possesses an additional RNA-dependent RNA polymerase motif. Both proteins are found in *in vitro* replication complexes isolated from infected plants (Watanabe et al., 1999).

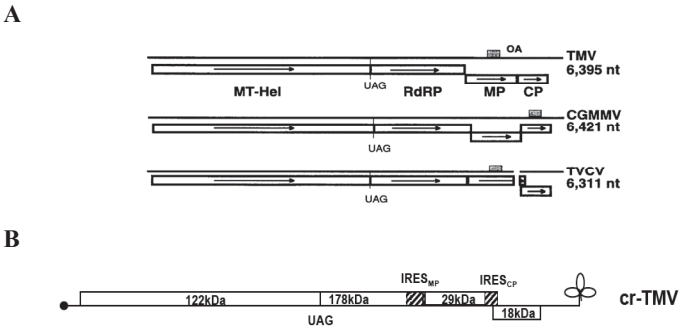

Figure 4: (A) Diagram of gene organization of tobamoviruses. The relative positions of methyltransferase-helicase (MT-Hel), RNA-dependent RNA polymerase (RdRP), movement protein (MP) and coat protein (CP) coding regions, the suppressible termination codons (UAG), and the origins of virion assembly (OA, stippled box) are indicated for representatives of subgroup 1 (TMV), subgroup 2 (CGMMV), and crucifer-pathogenic (TVCV) tobamoviruses. Gap in TVCV RNA and MP coding region indicates region of nucleotides missing relative to TMV (B) The genom organization cr-TMV. 122K and 178K replicase proteins, 29K movement protein (MP), 18K coat protein, IRES = Internal Ribosomal Entry Site, m^7Gppp cap is attached to the first nucleotide (guanylic acid) which is followed by an untranslated leader sequence of 69nt, 3' untranslated region fold in the terminal region to give a tRNA-like structure that aspects histidine.

Little is known about the precise mechanism of action of p122 upon infection. It has been demonstrated that the p126 protein of TMV OM strain (corresponding to the p122 of cr-TMV) forms a heterodimer replicase complex with p183 and two or more host proteins (Watanabe et al., 1999). Only one specific interaction between the p126 and p183 was identified, which is in the C-terminal half of p126 IR and N-terminal portion of p183 HEL domain (Goregaoker et al., 2001). The ratio between p126 and p183 is 1:1, although they are expressed in a 10:1 ratio during infection (Watanabe et al., 1999). The biological function of this excess amount of p126 remains to be determined. A substitution mutant of TMV-L strain in which the amber stop codon of p126 was replaced by tyrosine codon thus expressing only the p183 readthrough product was shown to replicate *in vivo* in the absence of the p126 protein. The growth rate of this mutant virus was about one-tenth of the rate of wild type (Ishikawa et al., 1986). All these results imply that ORF1 product besides being involved in the replication complex actions has other functions as well. Indeed the other analogous protein p130 of *Tomato mosaic virus* (ToMV) has been shown to have silencing suppressor function (Kubota et al., 2003). It was also shown that p130 does not suppress the activity of the pre-existing, sequence-specific silencing machinery, suggesting that p130 blocks the utilization of small RNAs in the formation of new effector complexes (Kubota et al., 2003), however, the molecular bases of the suppression remained to be determined.

Our objective was to find and characterize in detail the mode of action of cr-TMV silencing suppressor. We demonstrated that p122 protein has strong silencing suppressor activity and we explored the molecular mechanism of its silencing suppression. Our findings demonstrated that p122 prevents the si/miRNAs assembly into RISC complexes inhibiting the development of virus or transgene induced silencing activity. In contrast p122 has no effect on the slicer activity of preassembled RISC, both *in vitro* and *in vivo*. We also demonstrated that p122 interferes with miRNA-mediated pathways, however this interference is not a general effect instead it likely depends on the spatial and temporal coexpression of p122 and miRNAs.

In addition, based on this study and on an earlier study, we can conclude that the sequestration of small RNA molecules is a very effective and widespread strategy of plant viruses to counteract RNA silencing (Lakatos et al., 2006, Csorba et al., 2007).

Chapter III MATERIALS AND METHODS

III.1. Plasmid constructs

The full length infectious cDNA clone of the pUC19-cr-TMV was prepared previously (T. Dalmay unpublished results) and the pUC19-cr-TMV-Δp122 mutant virus clone has been prepared PCR mutagenesis substituting the amber stop codon of p122 (TAG) to the tyrosine codon (TAT). The p122 ORF was PCR amplified using appropriate primer pairs and cloned into pBIN61 binary plasmid and subsequently introduced into Agrobacterium and used for agroinfiltration assays. The following constructs used for different transient assays were described previously 35S-GFP, 35S-GFP-IR, 35S-sigma3 (Lichner et al., 2003), 35S-His-HC-Pro (Lakatos et al., 2006), GFP-Cym, GFP-PoLV (Pantoleo et al., 2007). GFP-171.1 and GFP-171.2 were kindly provided by O. Voinnet and were described previously (Parizotto et al., 2004). Construct 35S-p122-His was prepared amplifying the p122 ORF by PCR using a forward primer containing a start codon (italics) (5_ *ATG*GCACAATTTCAACAAACAATTGAC) and with a reverse primer containing RGS $(His)_6$ epitop codons (underlined) and the stop codon (italics) (5_*CTA*GTGATGGTGATGGTGATGCGATCCTCTTTGTATCCCCGCTTCAACTCTATAC ATGTC), and then this fragment cloned into SmaI-cleaved BIN61 vector. 35S-p122 was prepared as 35S-p122-His except that the His tag was omitted from the reverse primer. For Pri-miR171c construct we amplified the pri-miR171 sequence from cDNA of *A. thaliana* with the forward (5_TGAGCGCACTATCGGACATCAAATAC) and reverse primers (5_TAAACGCGTGATATTGGCACGGCTC), and cloned it into pBIN61-SmaI vector.

III.2. Virus constructs and plant inoculation

In vitro transcription of the pUC19-cr-TMV or pUC19-cr-TMV-Δp122 viral constructs from PmlI linearized DNA templates and the inoculation of RNA transcripts onto *Nicotiana benthamiana* and *A. thaliana Columbia-0* ecotype wild type or mutant plants were performed as described earlier (Dalmay et al., 1993) . In vitro RNA transcripts were capped with a cap analogue based on manufacturer‘s instructions (New England Biolabs, Hitchin, United Kingdom) and rub-inoculated on plant leaves (3 leaves/plant) with inoculation buffer (0,375 g glycine, 0,522 g K2HPO4, 1g

bentonite, 1g cellite in 100 ml buffer) in 1:1 ratio. After systemization of the virus plant material was collected for RNA and/or protein extraction (as described below).

III.3. Agroinfiltration assay

Transient expression of proteins is accomplished by infiltrating an *N. benthamiana* leaf of with a solution of *Agrobacterium tumefaciens* carrying a binary vector, in our case pBIN61, driving expression from a plant promoter. Proteins of interest are subsequently expressed in all cells of the infiltrated patch and can be assayed for function. If GFP is expressed it can be visualized by ultra-violet illumination.

A. tumefaciens C58C1/pBIN61 harboring the appropriate plasmid was infiltrated according to the method described previously (Silhavy et al., 2002). *N. benthamiana* was co-infiltrated with siRNA- or miRNA sensor constructs (OD600 = 0.15) and suppressor protein constructs (OD600 = 0.5). *N. benthamiana* GFP16c/RDRP6i line (Schwach et al., 2005) was co-infiltrated with 35S-GFP (OD600 = 0.1) or 35S-GFP-IR (OD600 = 0.4) (Silhavy et al., 2002) and suppressor protein constructs (OD600 = 0.5). pri-miR171c was infiltrated at OD600 = 0.4.

III.4. RNA extraction and „Northern blot“ analysis

Total RNA from agro infiltrated leaves or mock- and cr-TMV-infected systemic leaves was isolated using Trizol reagent (based on manufacturer‘s instructions, Sigma). RNA extraction was performed 4 dpi for *N. benthamiana* and 14 dpi for *A. thaliana*. The same total RNA extract was used for high and low molecular weight RNA Northern blot analysis as described in (Silhavy et al., 2002).

III.5. Protein extraction and „Western blot“ analysis

Infiltrated leaf tissues were homogenized 3 dpi in extraction buffer (150 mM Tris-HCl, pH 7.5, 6 M urea, 2% sodium dodecyl sulfate, and 5% _B-mercaptoethanol). Samples were boiled and cell debris was removed by centrifugation at 18,000 *g* at 4°C for 10 min. Supernatants were resolved on SDS-PAGE 8% and subjected to Western blot analysis. The proteins were visualized using anti-GFP, anti-His and anti-HA antibodies by chemiluminescence (ECL kit, Amersham) according to the manufacturer’s instructions. Commercially available antibodies were used for detection of erGFP,

6xHis-tagged proteins. Ponceau red staining was used to check the global protein content of the samples.

III.6. Gel mobility shift assay

For RNA binding reactions, labeled ssRNA or dsRNA (0.5 nM) were incubated with agrobacterium infiltrated *N. benthamiana* leaf extract containing ~1 µg total protein or the relevant dilutions. Binding reactions and the mobility shift assays were carried out as described (Merai et al., 2006).

III.7. Assay for siRNA distribution by gel filtration

Extracts were prepared from 0.5 to 1 g of systemic leaves of cr-TMV-infected or mock-infected *A. thaliana* in 0.5–1 ml buffer containing 30mM HEPES-KOH (pH 7.5), 100mM KCl, 2mM MgCl2, 1mM DTT and 5% glycerol. Band shift reactions were done by incubating the extracts with non-labelled synthetic siRNA for 30 min (as described by Merai et al. 2006) in 200 ml volume. The band shift reactions were chromatographed at 4°C on a Superdex-200 HR 10/30 column (Pharmacia) at 0.4 ml/min in a column buffer containing 30mM HEPES-KOH (pH 7.5), 100mM KCl, 2mM MgCl2, 1mMDTT and 5% glycerol. In all, 60 200 ml fractions were collected and used for RNA isolation. RNA molecules were separated on a 12% polyacrylamide and 8M urea containing sequencing gel and northern blots performed.

III.8. Native gel electrophoresis

Native gel electrophoresis for separation of silencing complexes was essentially as described in (Pham et al., 2004) with modifications. In direct competition assays, *in vitro* reactions that were used for target cleavage assays were incubated 30 minutes with 5 nM ^{32}P-labeled siRNA and suppressor protein, diluted with 10 µl of loading buffer (1"lysis buffer, 6% of Ficoll 400) and a part of it analyzed on a 3.9% (39:1 acrylamide:bisacrylamide) native acrylamide gel. Gels were dried, exposed to a storage phosphor screen and bands were quantified with a Genius Image Analyzer (Syngene).

III.9. The *Drosophila* heterologous system and the *in vitro* RNA silencing

Drosophila lysate preparation, target RNA labeling and siRNA annealing were described previously (Haley et al., 2003). In a 10 µl reaction, 2 µl of lysate and siRNA in 5 nM final concentrations were used in 1"lysis buffer containing 10% v/v of glycerol. GFP target RNA was *in vitro* transcribed with T7 polymerase in the presence of ^{32}P-UTP and used in 0.5 nM final concentration. In direct competition assays, reactions were incubated for 1 hour. In active RISC assays, siRNA and the extract were incubated for 30 minutes to allow RISC assembly, and then target RNA and suppressor proteins were added to the reaction. Samples were de-proteinized and RNA was analyzed on an 8% denaturing gel.

III.10. β-elimination assay

Periodate treatment and β -elimination were performed as previously described (Alefelder et al., 1998). RNA was dissolved in borax/boric acid buffer (0.06 M, pH 8.6) and sodium periodate (200 mM in water) was added to a final concentration of 25 mM. The RNA was then incubated in darkness at room temperature. After 1 h of incubation, 1/10 volume of glycerol was added to the RNA and the incubation was continued for an additional 30 min. The RNA was then precipitated in the presence of ethanol. For β elimination, the sodium periodate-treated RNA was dissolved in NaOH/borax/boric acid buffer (0.055 M, pH 9.5), incubated at 45 °C for 90 min, and precipitated with ethanol.

Chapter IV. RESULTS

IV.1. The cr-TMV replicase subunit protein p122 is a potent silencing suppressor protein *in vivo*

Earlier Kubota *et al.* (2003) reported that the ToMV p130 protein had silencing suppressor activity suggesting that homologous proteins in the closely related TMV species are also silencing suppressors. In the strain of TMV, which can infect *A. thaliana* (cr-TMV) this protein is encoded by the p122 gene (Figure 4B). Cr-TMV infected *A. thaliana* plant shows aberrant leaf development (Figure 5A, B) typical for plants carrying mutations in small RNA pathways – that supports the hypothesis that cr-TMV could have a silencing suppressor protein. To get insight in the nature of silencing suppressor activity of cr-TMV, previously described systemically post-transcriptionally silenced GFP transgenic *A. thaliana* (AmpxGFP and Amp) plants (Dalmay et al., 1993) were inoculated with cr-TMV. 14 days post inoculation (dpi) GFP fluorescence was assessed in the virus infected plants. Figure 5 C, D show that cr-TMV infection resulted in reversion of GFP expression.

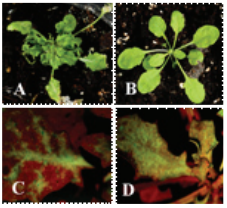

Figure 5: Infection of A. thaliana with cr-TMV leads to a strong phenotype after 2 weeks post infection (A) compared to uninfected plants (B). Reversion of silenced GFP in cr-TMV-infected transgenic Amp x GFP (C) and Amp (D) plants at 14 days post inoculation. GFP fluorescence was assessed in the plants under UV light using a dissecting microscope, and the photographs were taken 14 days after inoculation.

In the AmpxGFP plants were GFP-silencing is strong upon infection GFP silencing was suppressed only in and around the veins. These observations suggest that the suppressor protein of cr-TMV cannot suppress silencing completely in plants where silencing is strong.

To explore the molecular bases of silencing suppression of cr-TMV we tested the ToMV

p130 homologous in cr-TMV, the p122 protein and also the replicase larger subunit p178 protein (see later). We setted up an agroinfiltration assay: a modified non-tumorigen strain of *Agrobacterium tumefaciens* (C58C1) is used to deliver and promote the ectopical expression of a transgene in plant cells. The green fluorescence protein (GFP, the protein is fluorescent under UV light) is expressed in plant cells and the RNAi silence it after 2-3 day (see the model, Figure 6). The silencing of GFP is more robust if paralelly an inverted-repeat construct with homologous sequence (GFP-IR) is coexpressed. The IR is direct substrate of DCL, which produce high amount of siRNA leading ultimately to a very strong silencing effect. The silencing pathway is disrupted by viral silencing suppressor proteins, therefore this system can be used to monitor silencing suppressor activity of proteins.

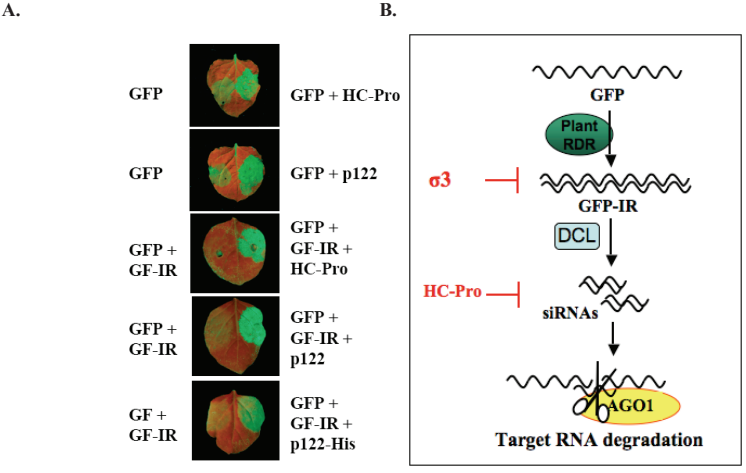

Figure 6: (A) Suppression of RNA silencing by p122. *N. benthamiana* leaves were agro infiltrated with 35S-GFP, 35S-GF-IR, 35S-HcPro, and 35S-p122, as indicated. The GFP fluorescence was monitored under UV light, and the photographs were taken 3 days after agroinfiltration. (B) A simplyfied model of viral suppressor mode of action on RNA silencing pathway. The place of HC-Pro and sigma3 inhibition of silencing is shown.

35S-p122 and 35S-p122-His were co-expressed with 35S-GFP or/and 35S-GFP-IR in leaves of *N. benthamiana* GFP16c/RDRP6i line (Schwach et al., 2005). The previously characterized HC-

Pro of *Tobacco etch virus* (TEV) that specifically binds to 21 nt siRNAs (Lakatos et al., 2006) and Reovirus sigma3 that exclusively binds to long dsRNA (Lichner et al., 2003) were used as controls. The transiently expressed p122 suppressed GFP silencing with the same efficiency as 35S-HC-Pro based on the bright fluorescence in the co-infiltrated areas (Figure 6). The suppression of GFP silencing by p122 was also very effective in the presence of 35S-GFP-IR expression (Figure 6A).

To confirm the visual observations we checked the GFP mRNA and GFP specific siRNA accumulation in GFP expressing line of *N. benthamiana* (16C). Total RNA samples were extracted from the infiltrated area 60 h after infiltration and analyzed by Northern blotting. In the presence of p122 or HC-Pro the GFP mRNA accumulated to a high level, even when 35S-GF-IR was simultaneously coexpressed, while the level of GFP mRNA was reduced when GFP was coexpressed with GF-IR in the absence of suppressors (Figure 7). A lower molecular weight band corresponding to the GF-IR transcript was observed only in the presence of sigma3 protein that inhibits DCL activity by dsRNA binding (Lichner et al., 2003) but not in the presence of HC-Pro or p122 (Figure 7, compare lanes 6,7 with 8 and lanes 14,15 with 16) indicating that p122 does not interfere with DCL similar to HC-Pro (see the model, figure 6), which was demonstrated previously (Lakatos et al., 2006).

As we expected, GFP siRNA accumulated in the leaf infiltrated only with GFP (Fig. 7, bottom, hybridized with GFP probe), while the presence of HC-Pro, p122, or sigma 3 proteins abolished the accumulation of GFP siRNAs. In contrast, when GFP was coinfiltrated with GF-IR, a very large amount of GFP siRNA accumulated in either the presence or the absence of p122 and HC-Pro (Fig. 7, bottom, lanes 5, 6, 7 and 13, 14, 15). The accumulation of siRNA was inhibited only in the presence of sigma 3, which compromises DCL activity (Lakatos et al., 2006). These results demonstrated, that p122 inhibits the accumulation of siRNA when the RNA silencing is triggered by sense GFP transcript. However, p122 did not inhibit the processing of GF-IR dsRNA into siRNAs but interfered with the silencing machinery downstream of siRNA generation. We also tested the effect of p122 on the accumulation of RDRP6-dependent secondary siRNAs (Moissiard et al., 2007). We have shown that p122 was able to inhibit the accumulation of secondary siRNAs detected by P-specific probe (Fig. 7, compare lane 1 to lane 3). The inhibition of secondary siRNA (P-specific siRNA) accumulation by p122 was very efficient regardless of whether the GFP silencing was triggered by GFP alone or GFP plus GF-IR expression (Fig. 7, compare lanes 1 to 3 and 5 to 7). It is worth noting that the majority of primary siRNAs (GF specific) were 24 nt long

and only one-third of the siRNAs were 21/22 nt long when the silencing was triggered by GF-IR (Fig. 7, middle, lanes 5, 6, 7 and 13, 14, 15 GF-specific siRNAs). In contrast, the majority of secondary siRNAs (P specific) were 21/22 nt long, suggesting that they were the products of DCL4 and DCL2, respectively. In the RDRP6- plants no secondary P-probe specific siRNA were observed proving that the signal was specific (Fig. 7, middle, lanes 13, 14, 15).

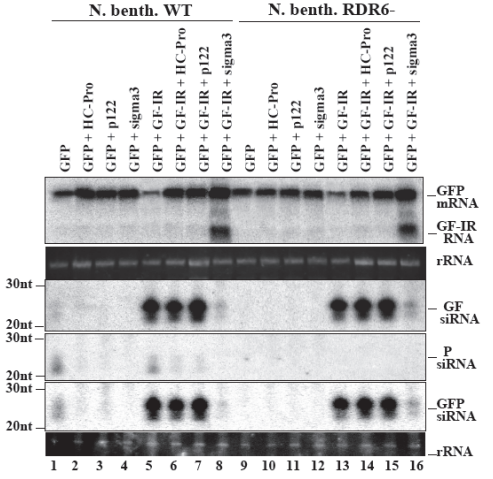

Figure 7: The p122 inhibits GFP RNA degradation but does not impair primary siRNA production. Leaves of the N. benthamiana GFP16C line were infiltrated as shown. For control we used RDRP6-mutant plant (Moissiard et al., 2007) extracts from infiltrated plants with the same construct or construct combinations respectively (lane 9 to 16). The RNA samples extracted 60 h after infiltration were subjected to Northern analysis using appropriate probes to detect GFP mRNA and GF-IR RNA and GFP-, GF-, and P-specific siRNAs. rRNA is shown as loading control.

We also analyzed the accumulation of virus-derived siRNA in cr-TMV-infected plants. Total RNA was extracted from *N. benthamiana* at 4 days post inoculation (dpi.) and from *A. thaliana* at 14 dpi. A large amount of 21nt long viral-siRNA accumulated in the virus-infected (Fig. 8), suggesting that p122 does not compromise the generation of viral siRNAs *in planta*.

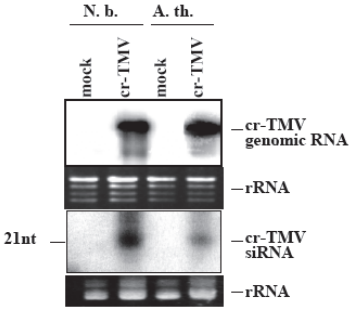

Figure 8: Accumulation of virus-specific siRNAs during cr-TMV infection. Total RNA was extracted from cr-TMV-infected *N. benthamiana* plants (N.b.) at 4 d.p.i. and *A. thaliana* plants (A.th.) at 14 d.p.i. and separated on denaturing agarose (for viral RNAs) and in 15% denaturing polyacrylamide gel (for siRNAs).

These results suggests that p122 acts downstream to DCL activity but upstream to RDRP-mediated siRNA amplification. Next we wanted to know whether p122 is able to interfere with RISC cleavage of target RNAs.

IV.2. p122 inhibits siRNA-directed RNA cleavage and the assembly of silencing-related complexes in *Drosophila* embryo extracts in a dose-dependent manner

The *Drosophila* embryo extract based RNA silencing system had been successfully used to characterize silencing suppressor proteins (Lakatos et al., 2006). This system allows the analysis of RNA-silencing complex formation and the cleavage activity of programmed RISC complex (Pham et al., 2004, Tomari et al., 2004). To better understand how p122 suppressor protein works we tested the siRNA programmed RISC activity in the presence of p122 protein. Repeated attempts to express p122 in bacteria were not successful therefore p122 was expressed in plants using binary expression vector (Merai et al., 2006).

To test p122 effect on RISC mediated cleavage we setted up two sets of reactions: a direct competition and an indirect competition assay (see the model, Figure 9B). In the direct competition assay the *Drosophila* embryo extract, the inducer siRNA, the labeled target RNA containing the sequence complementary to the inducer siRNA and the p122 containing plant extract (made from 35S-p122 agro-infiltrated *N. benthamiana* leaves at 3 days post infiltration) were added simultaneously into the reaction mix. After 30 minutes incubation RNA was phenol-chloroform-extracted and RISC activity quantified by measuring the amount of 5'-cleavage product (75 nt) of the target RNA (330 nt)(Figure 9, lanes 3-7).

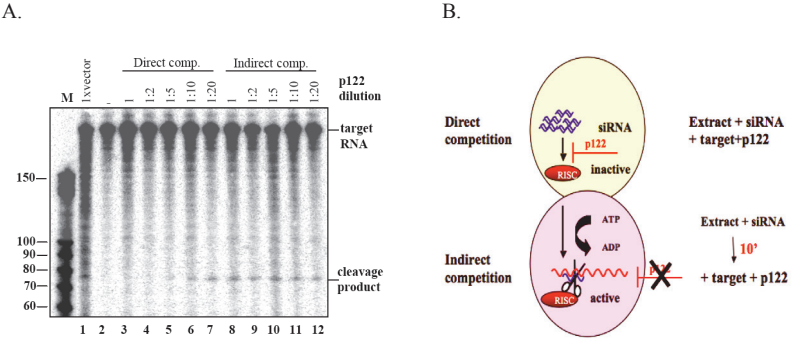

Figure 9: p122 protein inhibits siRNA-guided target cleavage. (A) p122 inhibited target cleavage *in vitro* in the direct-competition assay (lanes 3 to 7) but did not interfere with preprogrammed RISC activity (indirect-competition assay, lanes 8 to 12). In the direct-competition assay, *Drosophila* embryo extract, target RNA (0.5 nM), labeled siRNA (5 nM), and either empty-vector-infiltrated (lane 1) or p122-infiltrated *N. benthamiana* plant extracts were used at different dilutions, and all components were added simultaneously. In the indirect-competition assay, siRNAs were incubated with the *Drosophila* embryo extract for 10 min, and then target RNA and p122-infiltrated *N. benthamiana* plant extracts were added in different dilutions. The effect of p122 on RISC-mediated cleavage was monitored by the detection of cleavage products. Lane M shows RNA size markers; size is indicated in nucleotides. (B) The model of p122 action on RISC slicer activity.

We did a dilution seria for p122 (Figure 9A), for control reaction we used empty vector-infiltrated plant extract at the highest concentration used for p122 (lane 1), and we performed a reaction without embryo extract as negative control (lane 2). P122 was able to inhibit the target cleavage (Figure 9A lanes 3-5), likely preventing the assembly of RISC complex.

To analyze the effect of p122 on preassembled RISC activity (indirect competition) we pre-incubated the *Drosophila* embryo extract with inducer siRNA for 10 minutes and then the target and the suppressor containing plant extract or mock-infiltrated plant extract were added to the reaction and further incubated for 30 minutes (Figure 9, lanes 8-12). P122 proved to be a potent inhibitor of RISC-cleavage at higher concentrations (IC50 at 10 fold dilution) in the direct competition assay, but had no effect on preassembled RISC activity. Activity of programmed RISC was refractory to p122 suppressor protein regardless of the concentration. These results suggested that the siRNA-sequestration model may also explain the mechanism of p122 mediated suppression as was demonstrated for p19, HC-Pro and p21 (Silhavy et al., 2002, Vargason et al., 2003, Chapman et al., 2004, Lakatos et al., 2006).

IV.3. RISC formation is impaired *in vitro* in the presence of p122 protein

To test our hypothesis we analyzed the assembly of the RNA silencing complexes with siRNAs by electrophoretic mobility shift assay developed by Pham et al (2004) and adapted for suppressor protein assays by Lakatos *et al.* (2006). This technique is used to study the formation of the various stages of silencing complexes of *Drosophila* embryo (Figure 10).

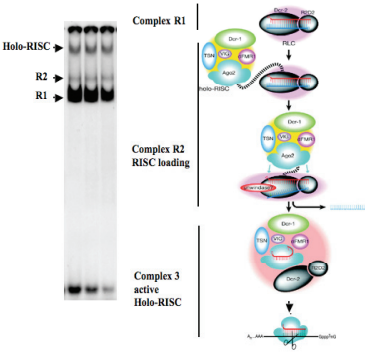

Figure 10. RNA silencing complex formation in *Drosophila* embryo extract system. (Pham *et al*. 2004).

P^{32}-labelled siRNAs and cleared native extract of 3 hours old *Drosophila* embryo are mixed together incubated for 30 minutes and resolved in 3.9% acrylamide gels. Three complexes can be detected: R1 (containing probably DCL and R2D2 proteins and ds siRNA), R2 (more proteins are attached, the siRNA is unwinded) and holo-RISC (the effector complex responsible for slicer activity) (see the model below).

As in the RISC cleavage assay were setted up two sets of reactions. In the direct competition assay we mixed inducer siRNA, Drosophila embryo extract and p122-infiltrated plant extracts simultaneously (Figure 11 lanes 3-8). In the indirect competition assay the embryo extract were pre-incubated with the labeled siRNA for 10 minutes and then p122-infiltrated plant extract was added at the same dilutions and further incubated (Figure 11 lanes 10-15). As control reactions we incubated embryo extract with labeled siRNA and empty vector infiltrated plant extract at the highest concentration used for direct and indirect competitions (lane 9 and 16).

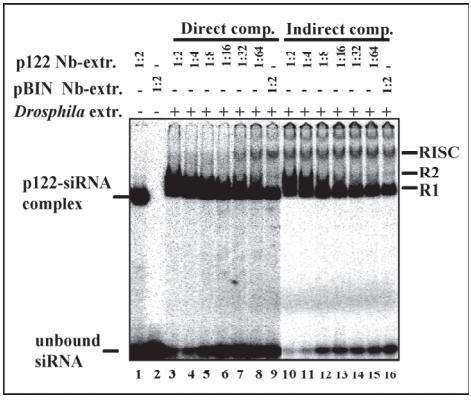

Figure 10: In vitro RISC formation is inhibited by p122 protein. The different forms of siRNA containing silencing-related complexes were separated in 3.9% native acrylamide gels. The positions of R1, R2, RISC, and p122-siRNAs are indicated. In the direct-competition (comp.) assay, Drosophila embryo extract (extr.), labeled siRNA, and pBIN empty-vector-infiltrated (lane 16) or p122-infiltrated (lanes 3 to 8) plant extract dilutions were added at the same time. In the indirect-competition reactions, embryo extracts were preincubated with labeled siRNA prior to the addition of the pBIN-infiltrated (lane 16) or p122-infiltrated (lanes 10 to 15) plant extracts. Control reactions were done with siRNA and pBIN-p122-infiltrated (lane 1) or siRNA and empty-vector-infiltrated plant extract (lanes 2).

In the direct competition experiment, p122 was able to inhibit the RISC-assembly up to 1:16 dilution of plant extract, while in the indirect assay p122 did not compromise the preassembled RISC in any dilutions. It is worth noting that a newly formed complex was observed running slightly above the R1 complex and also running faster than the siRNA-p122 complex (Figure 10, compare lane 1 with lanes 3 and 9 respectively).

The accumulation of this complex gradually diminished as the concentration of p122 decreased. We hypothesized that this was probably a p122-siRNA complex containing some unidentified cellular factors. These results and the observation of p122-siRNA formation strongly suggested that p122 was able to bind ds siRNAs and inhibited RNA silencing via siRNA sequestration.

IV.4. p122 does not inhibit siRNA- or miRNA- preloaded RISC activity *in vivo*

The *in vitro* data showed that p122 could not interfere with the preassembled RISC activity but prevented the new RISC complexes to be formed in *Drosophila* embryo system. To test the effect of p122 on the si/miRNA programmed RISC *in planta* we used GFP-based sensor systems developed previously (Lakatos et al., 2006, Pantaleo et al., 2007, Park et al., 2005).

N. benthamiana plants were infected with the p19 silencing suppressor mutant (Cym19stop) of *Cymbidium ringspot virus* (CymRSV) (Szittya et al., 2002) (Figure 12B). The wild type virus would kill the plant, but the p19 suppressor mutant virus titer is kept at a low level by the plant silencing system. At first virus symptoms are visible on plants (red arrow, Fig. 12A) but the plant recovers, the emerging leaves are with mild or no symptoms. These are called recovery leaves, the plant recovery plants. In the recovery leaves are present the virus-derived siRNA programmed RISC complexes, which are able to cleave the CymRSV specific sequences introduced into GFP sensor molecules (Pantaleo et al., 2007). The recovery leaves of Cym19stop-infected plants were infiltrated with the GFP-Cym-sensor construct and as negative control we used a GFP-PoLV sensor, which expresses a GFP mRNA containing a *Pothos latent virus* (PoLV) sequence at the 3' UTR (Figure 12C) (Pantaleo et al., 2007). This second GFP sensor is not expected to be silenced, because there are no RISC complexes programmed against the PoLV sequence.

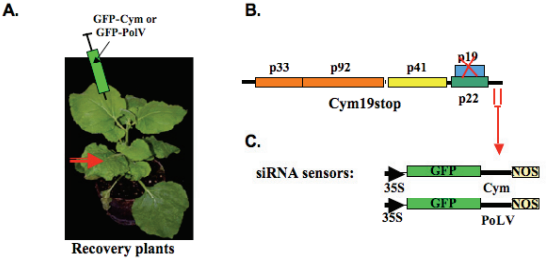

Figure 12: The GFP-virus sensor system used to monitor the activity of p122 silencing suppression on preloaded RISC. The Cym19stop (diagram of gene organization shown at right) infected recovery plants used for agroinfiltration of sensors (left). Cym19stop virus derived 3‘ terminal sequence used for construction of GFP sensor was cloned at 3‘ untranslated region of GFP ORF (right). A non-homologous sequence derived from Pothos latent virus 3‘ terminal part was used to build a control sensor which is not targeted in Cym19stop infected plants.

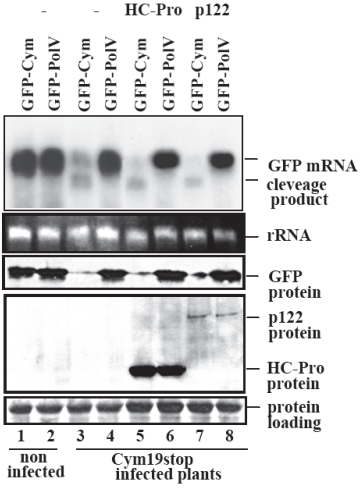

Figure 13: p122 protein does not inhibit preassembled siRISC activity in vivo. GFP-Cym or GFP-PolV sensor constructs were infiltrated (lanes 1,2 and 3,4) or coinfiltrated with HC-Pro (lanes 5,6) or p122 (lanes 7,8) into non-infected or Cym19stop-infected recovered leaves as indicated. GFP mRNA and protein were analyzed 3 days post infiltration in Northern blot and Western immunoblot assays.

Both GFP-Cym and GFP-PoLV were expressed at high level after agro-infiltration in the leaves of non-infected plants (Figure 13 lanes 1,2). However, when recovered leaves of Cym19stop infected plants were infiltrated, the GFP-Cym sensor mRNA was efficiently targeted by viral siRNA programmed RISC, reducing the level of sensor RNA and GFP expression. On contrary, the level of heterologous sensor Cym-PoLV remained high (Figure 13 compare lanes 3 and 4). The detection of 5‘ cleavage product of GFP-Cym further confirmed the activity of virus siRNA-programmed RISC. As we expected from in vitro experiments the co-expression of p122-His with the sensor GFP-Cym was not able to inhibit the cleavage of sensor RNA similarly to His-HC-Pro (Figure 13 lanes 5-8) and p19 (Pantaleo et al., 2007).

Viral siRNA and plant miRNA are very similar structures and share components of silencing pathways. Beside this cr-TMV virus infection cause severe symptoms similar to plants mutant in miRNA-pathway (Palatnik et al., 2003) or plants expressing silencing suppressors (Dunoyer et al. 2003). The viral symphoms could be at least partially atributed to disruption of miRNA pathways. Based on this, we wanted to know what is the effect of p122-His on miRNA loaded RISC activity.

A similar experiment was carried out using a cleavable sensor (GFP-171.1) and a non-cleavable mutant sensor (GFP- 171.2) constructs (Parizotto et al., 2004). The GFP-171.1 and GFP-171.2 constructs were infiltrated on the two sides of the same leaf, to ensure the same level of miR-171 loaded RISC and GFP fluorescence was monitored at 3 dpi.

Consistent to the previous results, the GFP mRNA and protein levels were reduced in the GFP-171.1 sensor infiltrated patch (Figure 14, lane 1) compared to the GFP-171.2 control (Figure 14 lane 2). This expression pattern was the same in the presence of p122 or the control HC-Pro (Figure 14 lanes 3-8). The expression of the suppressors was demonstrated by Western blot analysis.

These results strengthen our *in vitro* findings and demonstrate that p122 is not able to inhibit preloaded si- or miRISC activity *in vivo*, as observed in the *Drosophila* embryo system.
Based on this and the previous finding that p122 act downstream to DCL activity, we tested the siRNA binding capacity of p122.

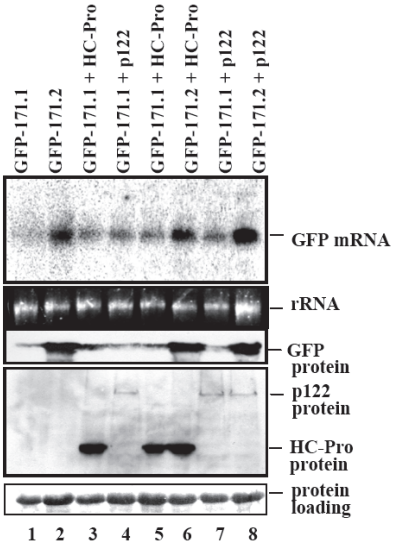

Figure 14: Preprogrammed miRISC activity is not affected by the presence of p122. The sensor construct GFP-171.1, bearing a perfect target site for miR171, is cleaved irrespective of the presence of the p122 protein. Not cleavable GFP-171.2 sensor, which bears a mutation in the target site, was used as negative control. For Western blot analysis, anti-GFP and anti-His antibodies were applied.

IV.5. The p122 protein binds double-stranded siRNAs in size-specific manner

To explore the affinity of p122 to siRNAs we carried out a detailed analysis of the RNA binding affinity of this protein. Electrophoretic mobility shift assays were performed using labeled synthetic single- or double-stranded RNA oligonucleotides in different sizes and diluted plant protein extract of 35S-p122 infiltrated *N. benthamiana* leaf (Figure 15). In these experiments, the previously characterized 35S-p19 of *Carnation Italian ringspot virus* (Lakatos et al., 2006) infiltrated *N. benthamiana* leaf extract was used as control (data not shown).

P122 did not show any single stranded RNA binding activity irrespectively of the length of

RNA (data not shown). The relative dissociation constant (K_r) of p122 for ds siRNA was in the same range as the p19-infiltrated plant extract (see Table 1.). p122 bound to 21nt RNA duplexes with 2nt 3'overhang with the highest affinity (Figure 15 A,C, squares). The size and the 3'overhangs of the siRNA proved to be important because the binding affinity was slightly reduced when 19 nt blunt dsRNAs (Figure 15 A,C, circles) or 21nt blunt dsRNAs (Figure 15 C, up triangles) were used, $K_{r/19} = 2$ and $K_{r/21} = 6$, respectively (K_r of p122 for 21 nt long siRNA is considered to be 1). The affinity for 24 nt siRNA species (Figure 14C, down triangles) was much lower: $K_{r/24} = 20$. P122 did not bind the other RNAs tested: 19nt dsRNA with 3' 2nt overhang 26nt dsRNA with 2nt 3'overhang, 49nt dsRNA (Figure 15 C) and 19-, 21-, 49nt single-stranded RNAs (data not shown). Since TMV siRNAs are 21 nt long (Figure 8), these results suggest a specific adaptation of the virus to counteract the antiviral silencing machinery.

sRNA form	**p122** dilution	**p122** K relative	**p19** Kd (nM)	**p19** K relative
19nt+2ov	0,015	**1**	0,17	**1**
ssRNA 21nt	-	NB	-	NB
ssRNA 24nt	-	NB	-	NB
ssRNA 49nt	-	NB	-	NB
19nt blunt	0,03	**2**	0,08	**0,47**
22nt+2ov	0,6	**21**	3,7	**22**
24nt+2ov	-	NB	12,7	**75**
47nt+2ov	-	NB		NB

Table 1: Comparison of tobamoviral p122 protein (based on this study) and tombusviral p19 protein (based on Vargason et al., 2003) RNA binding activity. *Bona fide* siRNA (19nt+2ov), single-strands 21nt, 24nt and 29nt RNA (ssRNA 21nt, ssRNA 24nt and ssRNA 49nt respectively), double-stranded 19 nt blunt RNA (19nt blunt), double-stranded 22nt, 24nt or 47nt long RNA with 2nt 3'overhangs (22nt+2ov, 24nt+2ov or 47nt+2ov respectively), not binding (NB). The table shows the Kd values of those RNA species which data were available in both cases.

To analyze the natural function of p122 during the course of virus infection, we tested the siRNA binding affinity of plant extract derived from wt cr-TMV infected plants. Figure 15E shows

that the plant extract from wt virus infected plants has the same binding activity as transiently expressed p122: it binds 21 nt siRNAs but does not bind 49 nt siRNAs.

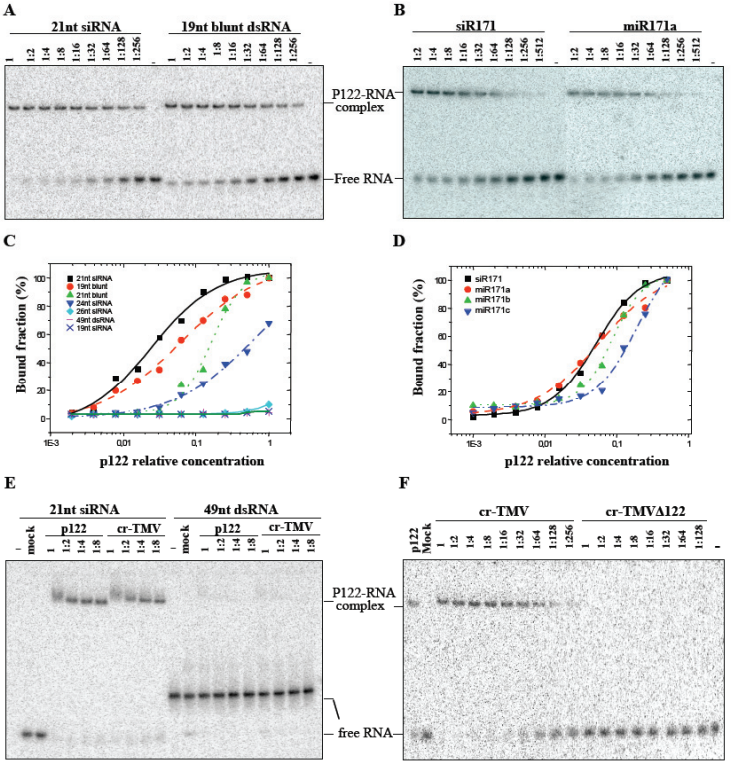

Figure 15: Affinities of p122 protein for different RNA duplexes. Twenty-one-nucleotide bona fide siRNA and 19-nt blunt-ended RNA duplexes (A) or 21-nt siRNA and miR171 duplexes (B) were incubated with a dilution series of p122-infiltrated plant extracts and loaded on a 5% native 0.5x Tris-borate-EDTA gel. (C) Determination of relative binding affinities of p122 extract for different RNA molecules. (D) Relative affinities of p122 protein for miRNA duplexes miR171a, miR171b, and miR171c compared with siR171. (E) Binding affinities for 21-nt siRNA and for 49-nt dsRNA using pBIN-p122-infiltrated or cr-TMV-infected plant extract. The complexes formed ran at the same mobility. Control reactions are shown without protein extract or with empty-vector-infiltrated plant extract. (F) Plant extracts infected with mutant virus not coding for p122 do not show any binding.

The mobility of p122-siRNA complex is the same as wt virus protein-siRNA complex suggesting that p122 is the only viral protein, which binds siRNA and the cr-TMV p178 viral replicase does not have siRNA binding activity. We were not able to test directly p178 for RNA binding because a 35S-p178-HA construct failed to express detectable amount of p178. However, indirect evidence suggesting that p178 did not bind siRNAs was obtained.

IV.6. P122 protein is the only suppressor of cr-TMV

To further test p178 protein suppressor activity, we created a mutant cr-TMV in which the amber stop codon of p122 was substituted by a tyrosine codon. The mutant virus (cr-TMV-Δp122) was able to replicate, although at lower rate than the wild type virus. To enhance the replication and systemization of the mutant virus we ectopically expressed p122 protein in the virus-inoculated leaves and quantified the mutant virus titer in the systemic leaves were p122 was not present.

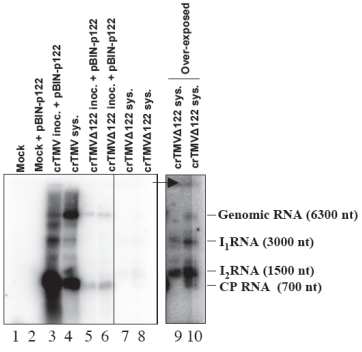

Figure 16: cr-TMV-Δp122 mutant virus infects *N. benthamiana* plants systemically at very low level. Systemization of the virus was enhanced by ectopical expression of p122 protein in the virus-inoculated leaves. Samples are indicated, lane 7 and 8 are pictured in an overexposed form in lane 9 and 10 respectively. The size of genomic RNA and subgenomic RNAs are shown at right. The arrow is pointed to the well.

The mutant cr-TMV-Δp122 virus was present in ~50 times lower amount compared to the cr-TMV wt virus (Figure 16). We compared the siRNA binding activity of the protein extract from cr-TMV-Δp122 infected plants with a serial dilution of wild type virus infected plant extract (Figure 15F).

64-times diluted (or even 128 times diluted) crTMV-infected extract was able to bind siRNAs, in contrast the cr-TMV-Δp122 infected plant extract did not show any siRNA binding activity (Figure 15F). This result suggests that the p178 readthrough product of p122 doesn't have siRNA binding activity and the p122 protein is solely responsible for anti-viral silencing upon cr-TMV infection.

In the next step we wanted to estimate the size of the p122-siRNA complex. We mixed synthetic P^{32}-labelled siRNAs with native extracts prepared from virus infected plant and incubated it for 30 min. The mix was subjected to gel-filtration chromatography on Superdex-200 column.

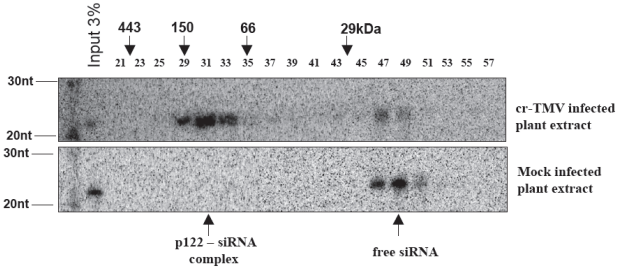

Figure 17: Synthetic siRNAs are in the higher molecular weight chromatography fractions of cr-TMV-infected plant extracts. The extracts prepared from systemic leaves of cr-TMV- or mock-infected *N. benthamiana* plants were size-separated by the Superdex-200 gel-filtration column, and then fractions were tested for the presence of siRNAs. RNA gel blots were probed with 32P-ATP labeled 21-nt synthetic GFP-derived RNA oligos. Decade size marker for RNA gel blots is shown at left. The elution position of protein molecular weight markers for both panels is shown above: 440 kDa, ferritin; 150 kDa, aldolase; 66 kDa, bovine serum albumin; 29 kDa, carbonic anhydrase. Molecular weight of p122-siRNA complex was estimated to be 108,4 kDa.

The synthetic siRNAs eluted in two peaks, mostly at about 120 kDa and a small fraction at free siRNA size. In the mock-infected plant extract all amount of siRNAs was in the free fraction. We approximated the size of the complex in the virus infected sample with interpolation to be 108,4 kDa (Figure 17). This is very likely to be the p122 monomer-siRNA complex size, as p178-siRNA

or p122-p178-siRNA complex would elute at much higher molecular weight. These findings unambigously exclude any involvement of p178 in the siRNA binding and therefore silencing suppression.

IV.7. The p122 binds miRNA duplexes and interferes with miRNA accumulation and 3‘-methylation

It is becoming clear that viruses not only suppress RNA silencing but the virus encoded silencing suppressors can also interfere with cellular functions that are controlled by plant miRNAs and endogenous siRNAs. This interference can contribute to viral symptoms as suggested previously for P1/HC-Pro of *Turnip mosaic virus* (Kasshau et al., 2003). However, considering our findings that p122 has a specific affinity to *bona fide* 21 nt ds-siRNA only in a narrow size range, it was not predictable whether p122 was able to inhibit miRNA pathways by binding ds miRNA intermediates or not, since these miRNA/miRNA* duplexes contain mismatches and bulges (regions of duplex which are not perfectly paired), which can modify the structure of miRNA duplexes compared to a perfectly matched siRNA duplex. To this end we tested p122 binding to synthetic miR171a, miR171b and miR171c RNA (Dharmacon) duplexes along with a miR171 siRNA having a perfectly matched star strand, in electromobility shift assay by making serial dilutions for p122 protein. We found that the binding affinity of p122 to the three miRNA/miRNA* (miR171a, miR171b, miR171c) duplexes are in the same range, (K_r=1.1, 1.6 and 1.9, respectively) and only slightly reduced as compared to the 171 siRNA perfect duplex. (K_r=1) (Figure 15B and D).

To explore the possible effect of p122 on miRNA pathways, *A. thaliana* Col-0 plants were infected with cr-TMV. After 14 days we observed severe mottling of systemically infected leaves and the edges of the leaves became serrated (Figure 5A) similar to those plants in which the miRNA pathways were compromised by mutation (Palatnik et al., 2003) or silencing suppressor proteins were expressed (Dunoyer et al., 2004).

This observation suggests that p122 may also interfere with miRNA pathways. We hypothesized that if p122 could bind miRNA/miRNA* duplexes *in vivo*, the accumulation level of miRNA* would increase because the miRNA duplexes would be stabilized by p122 binding,

similarly to the previously reported silencing suppressors, which bind miRNA duplexes (9, 24, 25).

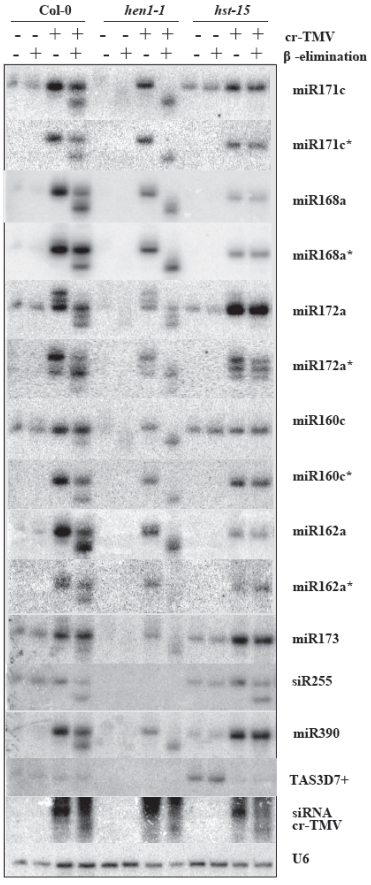

Figure 18: Accumulation and 3'-methylation of miRNAs and siRNAs upon cr-TMV infection. Elevated accumulation of the different miRNAs and miRNA*s have been observed in cr-TMV-infected plants. Total RNAs were extracted from cr-TMV-infected or mock-inoculated wt or mutant *A. thaliana* plants as indicated. For hybridization, labeled locked nucleic acid oligonucleotides complementary to the indicated miRNA and miRNA* were used. For virus-derived siRNAs and for U6 as a loading control, labeled complementary transcripts were applied as probes. For the identification of the methylation statuses of miRNAs and siRNAs, total RNAs from infected and non-infected plants were subjected (+) or not (–) to the ß-elimination reactions.

Indeed, upon cr-TMV infection both mature and star strands of the tested endogenous miRNAs (miR160c, miR162a, miR171c, miR168a, miR172) accumulated to a higher level compared to the mock-infected control plants (Figure 18). For example in the mock-inoculated samples the tested star miRNAs (miR160*, miR162a" miR171c*, miR172*) were under the detection level. This observations are in lane with our *in vitro* findings and strengthens the idea that p122 can interfere with miRNA pathways by sequestering miRNA duplexes and stabilizing them.

Interestingly, the accumulation of miR168 was particularly high in cr-TMV infected plants. This result prompted us to analyze the expression of its target AGO1 mRNA, which is controlled by miR168 since it was recently reported that the expression of miR168 and its target AGO1 are coregulated as consequence of AGO1 homeostasis (Vaucheret et al., 2006). In the *AGO1* mRNA 3‘ UTR there is a miR168 target site, so the *AGO1* level is controlled by miR168 in a negative feedback loop system. If more miR168-programmed RISC complex are present the faster will be the *AGO1* mRNA silencing, and ultimately less RISC will be assembled. As miRNA is stabilized in a duplex form by p122 protein *AGO1* mRNA cannot be downregulated. Indeed, the expression of *AGO1* mRNA in cr-TMV infected plants was elevated. The high level of AGO1 was neither influenced by the lack of methylation in *hen1-1* plant (Chen, 2005) nor in *hst-15* plants (Xie et al., 2005) where the miRNA nuclear export is affected (Park et al., 2005) (Figure 19) since the miR168 is extracted from the pathway by incorporation into p122 complex (in wt or *hen1-1*) and/or deficient transport from nucleus to cytoplasm (in *hst-15*).

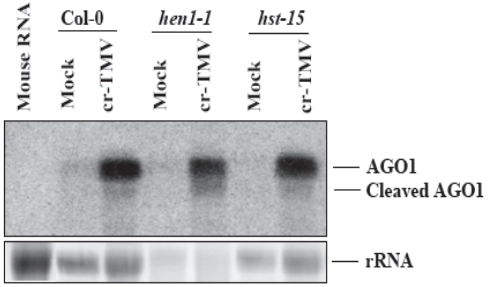

Figure 19: Northern analysis of AGO1 mRNA from mock- and virus-infected A. thaliana wt, *hen1-1 and hst-15* mutant plants as indicated. Mouse total RNA was used as control.

The miRNAs not only regulate mRNA levels but promote the accumulation of tasi-RNAs (see the model, Fig. 2), so it would be expected that the high amount of functional miR319 and miR173 will generate higher amount of TAS3D7+ and siR255, respectively. We found that miR319 and miR173 were unfunctional as the level of tasiRNAs was not elevated (Fig. 18). We conclude that probably the miRNAs in the cytoplasm are in p122-miRNA/miRNA* complex form.

We have also showed in our band shift experiments p122 recognized the 3‘ 2nt overhangs of siRNA duplexes predicting that p122 may interfere with 3‘ end methylation of endogenous small RNAs, miRNAs and viral siRNAs. Plant sRNAs are likely methylated by HEN1 methyltransferase at their 3’ terminal nucleotide at the 2’-hydroxyl group (Boutet et al., 2003, Yu et al., 2005). The methylation appears to protect them from oligouridylation and subsequent degradation (Li et al., 2005) and it is present in all species of small RNA family (siRNA, miRNA, tasiRNA, sense- and hairpin transgene derived, transposon- and repeat derived siRNAs). The methylation status of small RNAs can be assessed by treatment of the RNAs with sodium periodate followed by β-elimination. The free 2’OH groups are sensitive to the chemical modification and results in the elimination of the last nucleotide from the RNA. The resulting molecules will migrate faster in gel electrophoresis. To test whether p122 is able to interfere with the methylation of small RNAs, we checked the methylation status of several miRNAs and viral siRNAs generated upon virus-infection or in agroinfiltration transient assay. In virus infected *A. thaliana* leaf RNA extract the tested miRNAs showed different level of methylation as compared to the miRNAs derived from mock-inoculated plants (Figure 18). All miRNAs tested were fully methylated in mock-inoculated plants, while miRNAs - both mature and star strands - derived from cr-TMV infected plants were partially non-methylated, which was indicated the by the faster migration of miRNAs that underwent β-elimination (Figure 18). Interestingly, the methylation of miR160c, miR171c and miR168a* were only little effected in the presence of cr-TMV.

The partial inhibition of miRNA and viral siRNA methylation in the virus infected plants were somewhat surprising, since HEN1 suggested to be localized in the nucleus, while cr-TMV replicates exclusively in cytoplasm (Hull, 2002). Furthermore, we can rule out the existence of other unidentified methylase, which may operates in the cytoplasm since none of the miRNAs and

siRNAs were methylated in the in *hen1-1* plants. These findings suggested that HEN1 is active both in the nucleus and in the cytoplasm. Moreover, the ability of virus to interfere with miRNA methylation may suggest that miRNAs exported from the nucleus to the cytoplasm are in both methylated and non-methylated forms. To better understand which is the compartment where the methylation of miRNAs and siRNAs occur *hst-15* plants - in which the miRNA nuclear export is affected (Park et al., 2005) - were also infected with cr-TMV. The virus infection resulted in elevated accumulation of all tested miRNA including both mature and star strands. More importantly these miRNAs were completely methylated suggesting that the virus infection could not inhibit the methylation of miRNAs. This finding is likely the consequence of the fact that the nuclear export of miRNAs to the cytoplasm is compromised. In contrast to miRNAs the methylation of virus siRNAs were partially inhibited similarly to wt plants since they were present in the same compartment where the virus replicated. This result further supports the HEN1 activity in the cytoplasm.

We have shown that p122 efficiently binds ds siRNAs and miRNA intermediates, which strongly suggests that the small RNA binding activity of p122 is responsible for the inhibition of small RNA methylation. To further support our hypothesis p122 was coexpressed with 35S-pri-miRNA171c hairpin (that generates miRNA171c duplexes) using agroinfiltration (Figure 20B). As a control 35S-pri-miRNA171c was also co-infiltrated with 35S-HC-Pro or empty vector. When 35S-pri-miRNA171c was co-infiltrated with the empty vector the generated miR171c mature and miR171c* RNAs were resistant to the B-elimination treatment. It is worthy to note that the amount of miRNA in the infiltrated patch is in high excess as compared to the natural miRNA levels, but even in this case the methylation was 100% (Figure 20B lanes 3,4), suggesting that this step in miRNA biogenesis is very efficient. Importantly, B-elimination reaction was complete as demonstrated by the synthetic oligonucleotide controls (Figure 20 lanes 9-12). The co-expressed p122 partially inhibited miR171c methylation (Figure 20B lanes 7,8) similarly to virus-infected plants (Figure 18) and to HC-Pro (Figure 20B lanes 5,6). Interestingly, the miR171c* strand was fully methylated in p122 infiltrated plants, suggesting that one of the two termini of miR171c/miR171c* is not protected by p122 and it is accessible for HEN1 mediated methylation.

We also tested the effect of p122 on transgene-derived siRNAs (Figure 20A) and found that

p122 was able to interfere mostly with the methylation of 21nt long species, affected only very slightly the methylation of 22nt siRNA and did not interfere with the methylation of 24nt siRNA (Figure 20A, lane 6). Our finding that p122 binds 24 nt siRNAs with much lower efficiency than 21 nt siRNA could explain why p122 did not block the methylation of 24 nt siRNAs.

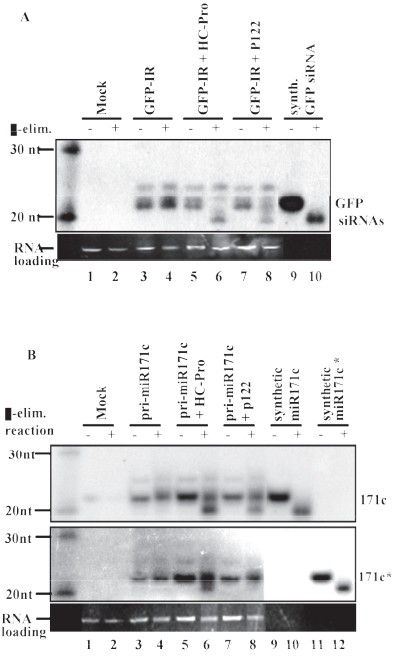

Figure 20: Effect of p122 protein on the methylation of GFP-derived siRNA and overexpressed miRNA 171c. Total RNA extracts were subjected (+) or not (–) to ß-elimination reactions, and the blots were hybridized as for Fig. 6. (A) GFP-IR-derived siRNAs are fully methylated in the absence of suppressor proteins. HC-Pro completely inhibited the methylation of 21-nt-long siRNAs and partially inhibited the methylation of 22-nt siRNAs (lane 6). p122 protein partially interfered with the methylation of 21- and 22-nt-long siRNA species (lane 8). Neither suppressor blocked the methylation of 24-nt siRNA molecules (lanes 6 and 8). Nonmethylated synthetic RNA oligonucleotides were used as positive controls for ß-elimination reactions (lanes 9 and 10). (B) Overexpressed miR171c and miR171c* are fully methylated in the absence of silencing suppressors (lane 4). HC-Pro and p122 proteins partially inhibited the methylation of miR171c (lanes 6 and 8) but not that of miR171c*. Positive controls for the ß-elimination test are shown in lanes 10 and 12.

Chapter V. DISCUSSION

The p122 protein of cr-TMV is the homologous gene of the previously reported p126 protein of TMV, which is the subunit of viral replicase complex (Hull, 2002). The p126 forms complex with the p183 protein, the readthrough product of p126, at a one to one ratio (Watanabe et al., 1999). However, it was also shown that p126 expressed approximately in 10 times molar excess to p183 suggesting that the protein has other important functions in the virus life cycle (Watanabe et al., 1999). Indeed, our finding demonstrated that the p122 of cr-TMV is also functioning as a silencing suppressor protein apart from its replicase function. The combination of replicase and the silencing suppression functions of the same protein are likely to be advantageous for the virus, since the replicating RNA is probably the most exposed form of the viral genome to silencing mediated RNA degradation. In this work we present an extensive characterization of silencing function of cr-TMV p122 replicase protein.

V.1. The p122 protein of cr-TMV efficiently suppresses RNA silencing by siRNA sequestration

The analysis of small RNA content of virus infected plant and the results of agroinfiltration assay suggested that p122 did not inhibit Dicer activity, since both viral and GFP-IR derived siRNAs accumulated to high level. Our *in vivo* and *in vitro* studies also showed that p122 acts upstream to RISC programming step, since the loaded siRISC and miRISC cannot be inhibited by the presence of the p122 protein. This feature resembles to the ToMV suppressor p130, which had been suggested to suppress GFP silencing but cannot interfere with the pre-programmed RNA silencing machinery (Kubota et al., 2003). Our results demonstrated that molecular bases of silencing suppression by p122 is the sequestration of siRNAs thus preventing the assembly of siRNA containing effector complexes in the antiviral silencing response. By sequestration of miRNA duplexes p122 interferes with endogenous pathways this phenomenon probably contributing to the virus symptoms development upon infection. The impact of p122 on antiviral-RISC assembly is immediate but delayed in the case of miRNA or endogenous siRNAs, since is not able to inhibit

loaded RISC activity.

In addition we have shown that p122 size selectively binds *bona fide* siRNA similarly to p19 of tombusviruses and HC-Pro of TEV (Lakatos et al., 2004, Vargason et al., 2003, Merai et al., 2006), (see Table 1.). This finding further supports the model of siRNA-RISC mediated targeting of viral RNA (Pantaleo et al., 2007, Zhang et al., 2006) in contrast to the theoretical possibility that Dicer activity alone could control the efficient viral invasion.

Since p122 is part of replicase complex it is interesting that p122 does not bind 49nt long double-stranded or single-stranded RNA molecules. However, it is possible that in the replicase complex p122 has different RNA binding activity or another component of the viral replicase complex, p178 protein or the p122-p178 heterodimer binds the replicating long viral RNAs. Is also possible that a plant protein modulates the RNA binding affinity of the replicase components.

We present indirect evidences that p122 protein is the solely silencing suppressor protein upon cr-TMV viral infection, and forms a protein-siRNA complex probably in a monomer form, in contrast to p19 (Vargason et al., 2003) or HC-Pro (Plisson et al., 2003).

V.2. Cr-TMV infection resulted in the elevated accumulation of miRNAs

P122 efficiently binds ds miRNA intermediates *in vitro* and the simultaneous accumulation of miRNA/miRNA* in cr-TMV infected plant suggesting that p122 binds miRNA duplexes also *in planta*. The symptoms of cr-TMV infected plants may indicate that the miRNA pathways are compromised. The *in vitro* band shift assay demonstrated that the miRNA mismatches did not alter significantly the binding affinity of p122 protein to miRNA duplexes, which is in line with the finding that the accumulation of tested miRNAs and miRNA*s were increased in virus infected plants. It is not clear whether this is due to stabilization of the miRNA duplex in the miRNA-p122 complex or the large amount of viral siRNAs alter the synthesis-degradation equilibrium of miRNA biogenesis. The miRNAs are produced by DCL1 protein, which is regulated through a feedback loop by miR162 RNA (56). Elevated level of *DCL1* mRNA was detected in *dcl1* and *hen1-1* mutant plants, or in plants expressing a viral suppressor TuMV P1/HC-Pro, which inhibits miRNA-guided degradation of *DCL1* mRNA (Xie et al., 2003). In cr-TMV infected leaves both miR162a and

miR162a*, similarly to other miRNA/miRNA* pairs, accumulated in virus infected plants, and their methylation was partially blocked suggesting that p122 binds miR162a/miR162a* duplexes *in vivo*, which can lead to stabilization and accumulation of these miRNAs. p122 may block the down-regulation of *DCL1* mRNA by miR162 resulting in higher level of DCL1, which ultimately responsible for elevated processing of all miRNAs. Another possibility is that viral siRNAs present in a large amount block miRNA degradation by overloading the small RNA degradation pathway. miR168a and its corresponding target AGO1 accumulated particularly high level during the development of virus infection, suggesting that AGO1 homeostasis (Vaucheret et al., 2006) keeps the balance between the miR168 and AGO1 expressions.

V.3. P122 inhibits the 3'methylation of small RNAs

Previous findings demonstrated that transgenically expressed silencing suppressor proteins, which are able to bind siRNAs inhibit 3'-methylation (Yu et al., 2005). In addition, inhibition HEN1 mediated methylation of small RNAs has been observed in *Oilseed rape mosaic virus* (ORMV) infected *A. thaliana* plants (Blevins et al., 2006). Our findings clearly indicated that the methylation of viral siRNAs, transgene derived siRNAs and miRNAs is inhibited by p122 likely through the binding of mi/siRNA duplexes. Our results also demonstrated that HEN1 operates both in the nucleus and cytoplasm since cr-TMV, as many other positive strand RNA viruses, replicates in cytoplasm and the virus derived siRNAs were partially methylated (Figure 18). Importantly, we can exclude the activity an other not unidentified methylase, which may operates in the cytoplasm since all miRNAs an siRNAs were not methylated in the in *hen1-1* plants. The ability of the replicating virus to interfere with miRNA methylation suggests that miRNAs are exported from the nucleus to the cytoplasm in both methylated and non-methylated forms. The infection of *hst-15* plants - in which the miRNA nuclear export might be compromised - resulted in the elevated accumulation of all tested miRNA and these miRNAs were completely methylated. This demonstrated that the virus infection could not inhibit the methylation of miRNAs, likely because they were separated in different compartments while the methylation of virus siRNAs were partially inhibited (as in wt plants) since they were present in the same compartments where the

virus replicated. Indeed the activity of HEN1 in the cytoplasm was supported by a recent report, which demonstrates that HEN1 is present both in the nucleus and the cytoplasm (Fang et al., 2007).

Another puzzling result is the asymmetrical methylation of the miRNAs upon virus infection. In some cases the mature strand is protected by the methyl group and the star strand is not (miR159/mir159*, not shown), while in other cases we observed the opposite situation where the miR171c methylation was partially blocked but the miR171c* was not (Figure 20). The asymmetrical methylation could be a consequence of two step kinetics of the HEN1 enzyme. In this scenario the suppressors binds the miRNA after the first step in which one of the strands is methylated. It is also possible that the p122-miRNA/miRNA* complex is not symmetrical, therefore HEN1 and/or p122 recognize the ends of miRNA duplexes asymmetrically. The difference between the suppression of methylation of different species could be due to a spatial and temporal compartmentalization of the different miRNAs and p122 protein. We observed that the transgene derived 21nt siRNAs were sensitive to the B-elimination reaction in the p122 infiltrated tissue indicating that they were not methylated, while 24nt siRNAs were fully methylated.

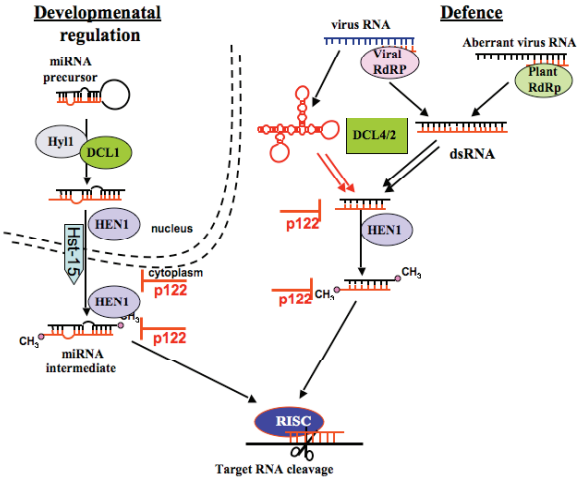

Figure 21: The model of silencing suppression mechanism of p122 protein.

These results are likely the consequence of the preferential binding of 21nt siRNAs versus 24nt by the p122 suppressor protein further supporting our finding that p122 binds siRNA duplexes in a size specific manner.

In conclusion, the multifunctional p122 protein of cr-TMV is a very potent silencing suppressor protein, which blocks the intermediate step of antiviral and endogenous silencing pathways preventing the assembly of DCL enzymes generated si/miRNA duplexes into effector complexes (Figure 21).

V.4. The siRNA binding is a widespread and effective strategy for viral silencing suppressors

Many silencing suppressor proteins are evolutionary unrelated, and different molecular mechanisms were proposed for their activity. HC-Pro was one of the first viral proteins identified as a suppressor of transgene- and virus-induced RNA silencing. Analyses of data from variant experimental systems led to the development of several different models for the mechanism of HC-Pro silencing suppression. In one model, HC-Pro was proposed to reverse established RNA silencing (Anandalakshmi et al, 1998; Brigneti et al, 1998; Voinnet et al, 1999; Llave et al, 2000). Another model involved the enlistment of a cellular negative regulator of RNA silencing, such as rgs-CaM, a calmodulin-related protein (Anandalakshmi et al, 2000). A third model proposed that HC-Pro acts downstream of an RDRP, but inhibits accumulation of siRNAs, suggesting that DICER activity was impaired (Mallory et al, 2001; Dunoyer et al, 2004). A fourth model predicted that RISC activation was suppressed through interaction between HC-Pro and a protein or complex required for siRNA duplex unwinding (Chapman et al, 2004). Importantly, most comparative studies concluded that the possible mechanism by which HC-Pro suppresses RNA silencing differs from the mechanism of other suppressor proteins, including p19 of tombusviruses and p21 of BYV (Chapman et al, 2004; Dunoyer et al, 2004; Voinnet, 2005). Detailed studies demonstrated that the molecular basis of silencing suppression of p19 protein of tombusviruses and p21 of Beet yellows virus (BYV) is siRNA sequestration (Silhavy et al, 2002; Vargason et al, 2003; Chapman et al, 2004; Dunoyer et al, 2004; Lakatos et al, 2004).

To establish a more detailed model for the molecular basis of RNA silencing suppression by these suppressor proteins, we used different approaches for their comparative characterization. This included *in vitro* and *in vivo* approaches to explore the molecular mechanisms by which p19, p21 and HC-Pro interfere with RNA silencing machinery. We presented evidence that all three silencing suppressors are dsRNA-binding proteins that interact physically with siRNA duplexes *in vivo* as well as *in vitro* (Lakatos et al., 2006). We also demonstrated that, similar to p19, HC-Pro and p21 inhibit siRNA-directed target RNA cleavage in the *Drosophila in vitro* RNA silencing system. Moreover, p19, HC-Pro and p21 uniformly inhibit the siRNA-initiated RISC assembly pathway by preventing RNA silencing initiator complex formation through siRNA sequestration (Lakatos et al., 2006).

It is tempting to speculate that the siRNA binding is one of the most effective strategies to inhibit silencing. As most of the viruses can infect more than one plant the RNA-binding is more reliable strategy than the targeting of silencing proteins, which can widely vary from one species to the other, although there are also counter-examples like 2b (Zhang et al., 2006) or p0 (Baumberger et al., 2007, Bortolamiol et al., 2007) silencing suppressors. Many dsRNA-binding suppressors are evolutionarily unrelated suggesting that dsRNA-binding silencing suppressors have evolved independently many times in convergent evolution, resulting in remarkably similar dsRNA-binding characteristics (Table 1, Lakatos et al., 2006, Merai et al., 2006). After the production of siRNAs in the silencing pathway this molecules are promptly sequestered and blocked to incorporat into the effector complex or to take part in the amplification processes mediated by the plant RDRP proteins.

In the same time the virus replication strategy evolved in such a way, not to affect as much as possible the endogenous pathways. Because the miRNA duplex intermediates resemble very much to the siRNAs, the suppressors bind them, blocking the loading of new RISC complexes, by this contributing to viral symptom development. Very important to remark that this strategy do not affect already programmed RISC complexes, which provides a delayed impact on miRNA pathway. We showed that none of these silencing suppressors (p19, p21, HC-Pro or p122) inhibit preassembled si- or miRISC activity *in vitro* or *in vivo*.

Chapter VI OUTLOOK

There are viruses, which use other strategies for suppressing RNA silencing. The *Beet Western Yellows Virus* silencing suppressor protein P0 inactivates Argonaute 1 (Ago1) effector protein of silencing, leading to its degradation in a proteasome-independent manner (Baumberger et al., 2007, Bortolamiol et al., 2007). The precise molecular mechanism of P0 action is still not known exactly. We tested P0 suppressor activity on pre-programmed silencing activity *in vivo*. Like potyviral suppressor HC-Pro, P0 could not interfere with miRNA- or siRNA-programmed silencing activity (unpublished data). This suggests that P0 acts against Ago1 before miRNA/siRNA loading. In transient assay the presence of P0 suppressor Ago1 protein level was strongly diminished but not Ago1 mRNA level. However if coinfiltrated with GFP-inverted-repeat (IR) construct, which overloads the system with GFP siRNAs, Ago1 protein level in the presence of P0 was elevated. The protective effect of siRNAs on Ago1 protein can be followed also on plant silencing complexes. P0 blocks the formation of plant silencing complexes but GFP-derived siRNA are able to revert this effect (unpublished data). This provides a mild effect on Ago1-mediated endogenous pathways and in the same time an efficacious and instant inhibition of anti-viral silencing.

An obvious follow-up would therefore be the precise biochemical characterization of P0 silencing suppressor protein with respect to his interaction and degradation effect on unloaded form of Ago1 protein. This would further demonstrate that the bottleneck of silencing pathway is the incorporation of the siRNA into amplification and/or effector complexes, and P0 targets exactly the same key step as the siRNA-binding suppressors neutralizing the protein but not the RNA counterpart of „minimal-RISC“ (Csorba et al, 2009 manuscript).

PROPOSITIONS

The replicase subunit protein of cr-TMV efficiently suppresses RNA silencing by siRNA sequestration but cannot interfere with the programmed silencing complexes *in vivo* (Csorba et al. 2007)

Upon cr-TMV infection p122 is the solely effector silencing suppressor protein (this thesis).

Cr-TMV infection elevates the level of miRNA duplexes by sequestering them and block their downstream functions (Csorba et al. 2007).

P122 inhibits the 3'methylation of small RNAs (Csorba et al. 2007)

The siRNA binding is a widely used strategy for viral silencing suppressors (Lakatos et al., 2006)

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Crucifer-infecting *Tobacco mosaic virus* replicase protein p122 is an RNA silencing suppressor

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PH. D. THESIS SUMMARY

RNA silencing (also known as RNA interference) is a general term for a particular collection of phenomena in which short RNA molecules trigger repression of homologous sequences. It is a highly conserved pathway, found in a large variety of eukaryotic organisms, and its main characteristic is the use of small RNA molecules of 21–25 nucleotides that confer high specificity to the target sequence.

One of the functions of RNA silencing in plants is to defend against molecular parasites such as viruses, retrotranspozons, and transgenes. Plant viruses are inducers as well as targets of RNA silencing based antiviral defense. Replication intermediates or folded viral RNAs activate RNA silencing generating small interfering (si) RNAs, which are the key players in the antiviral response. Viruses are able to counteract RNA silencing by expressing silencing suppressor proteins. It has been shown that many of the identified silencing suppressor proteins bind long dsRNA or siRNAs and thereby prevent assembly of the silencing effector complexes. In this study we have shown that the 122 kDa replicase small subunit (p122 protein) of crucifer-infecting *Tobacco mosaic virus* (cr-TMV) is a potent silencing suppressor protein. We found that the p122 protein does not interfere with Dicer-mediated primary siRNA production but preferentially binds to double-stranded 21nt siRNA and micro (mi) RNA intermediates having 2 nt 3'overhangs. By this p122 inhibits the incorporation of siRNA and miRNA into silencing related complexes both *in vitro* and *in planta*, and on the other hand, blocks the RNA-dependent RNA polimerase-mediated secondary siRNA production. P122 cannot interfere with the previously programmed RISC complexes *in vivo* and *in vitro*. In addition, our results also suggest that the virus infection and/or sequestration of the siRNA and miRNA molecules enhance miRNAs accumulation despite preventing their methylation.

The strategy of p122, not to affect already programmed RISC complexes, provides a delayed impact on miRNA pathway but an immediate and robust antiviral silencing suppression.

A keresztesvirágúakat fertőző dohány mozaik vírus replikáz alegysége, a p122 fehérje RNS silencing szupresszor

Csorba Tibor Levente

Doktori PhD értekezés összefoglaló

Az RNS interferencia (RNA interference, RNAi) az eukariótákban konzervált jelenségcsoport, mely során a nem transzlálódó RNS molekulák negatív regulátor szerepet töltenek be a sejt fehérjekészletének poszttranszkripciós szintű szabályozásában. Az RNAi szerepet játszik egyrészt a génregulációban, stresszválaszokban másrészt a genom védelmét szolgálja önző genetikai elemek (transzpozonok, transzgének) és vírusok inváziójával szemben.

Már a korai vizsgálatok rámutattak arra, hogy az RNAi egyik fő feladata a vírusfertőzések elleni védelem. Az RNAi felismeri a sejtben megjelenő erős másodlagos strukturákat hordozó vírus RNS-t, vagy a kétszálú vírus replikatív intermediereket és egy endoribonukleáz család enzimeinek, a Dicer-eknek a segítségével kis interferáló RNS-ekké (siRNS) érleti ezeket. A jellegzetes strukturával (21-24 nt hosszú, duplaszálú, 2nt 3'túlnyúló végű RNS) rendelkező siRNS-ek kitekeredésük után az RNS-indukálta silencing komplexbe (RISC) épülnek és szekvenciaspecifikusan vezérlik a homológ RNS-ek hasítását vagy transzlációs gátlását. A siRNSek az RNS-függő RNS polimeráz (RdRP) komplexekbe is beépülhetnek, mely másodlagos siRNSek keletkezéséért felelős egy amplifikációs folyamat során. A vírusok olyan szupresszor fehérjéket (silencing szupresszorok) kódolnak, melyekkel inaktiválják az RNAi alapú védekezési reakciót.

Munkánk során a keresztesvirágúakat fertőző dohány mozaik vírus (crucifer-infecting *Tobacco mosaic virus*, cr-TMV) silencing szupresszorát azonosítottuk és jellemeztük. A vírus replikáz kis alegysége, a 122 kDa (p122) fehérje hatékonyan gátolja a növényi védelmi rendszert. A p122 erős affinitást mutat a virális siRNS-ek és endogén kis RNS-ek (mikró RNS-ek, miRNS) iránt *in vitro*. Nem befolyásolja sem az elsődleges siRNS érést, sem a programozott RISC aktivitást *in vivo* és *in vitro*. A siRNSek és miRNSek megfogása által hatékonyan képes gátolni egyrészt az új siRNS- és miRNS-programozott RISC komplexek felépülését, másrészt az RdRP komplexekbe való beépülést és az ezt követő másodlagos siRNS amplifikációt.

Ez a stratégia azonnali gátlást biztosít az antivirális silencing tekintetében, de késleltetett hatású az endogén silencing útvonalakra nézve. Ennek jelentősége lehet a vírus evolúciós sikerének fokozásában.

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